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FINAL REPORT

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DEVELOPMENTAL COMPETENCE OF OOCYTES IN CATTLE

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Developmental Competence of Oocytes in Cattle
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Final Report

(a) **Abstract/Summary** - Maximum of one page in lay language. This must include project objectives, results, and conclusions for use in publications and in the Department database

Livestock producers and scientists alike have come to accept a certain level of *natural* reproductive inefficiency. The nature of this inefficiency is unknown, but must inevitably relate to the capability of the male and female germ cells (sperm and egg). Inefficiency in detecting or eliciting oocyte (egg) competence represents a fundamental cause of reproductive inefficiency in livestock regardless of whether the production system is simple (e.g., natural breeding) or highly technical (e.g., advanced reproductive technologies). The **long-term objective** is to improve reproductive efficiency by developing ways to diagnose and induce oocyte competence. The ovarian follicle and the oocyte it contains are in intimate structural and functional contact with each other, and have a metabolic dialogue that is only vaguely understood. The fundamental notion upon which this proposal is based is that the *functional status of the follicle influences the developmental competence of its oocyte*. The **short-term objectives** of this project were to determine the influence of follicular and hormonal status on the competence of the oocyte to develop into an embryo. Experiments were designed to determine the effects of the follicular phase (Study 1), progesterone (Study 2), and endogenous or exogenous LH on the developmental competence of the oocyte (Study 3). Knowing factors that affect the developmental capability of oocytes will make it possible to explain infertility observed in the field and provide a means by which fertility can be improved.

(b) **Introduction** - Brief project background, rationale and objectives

If fertilization rate exceeds 90% after a single breeding in cattle and in horses, why is pregnancy rate only 60% at the time of diagnosis a month later? Why do pigs and sheep have 15% fewer conceptuses than ovulations? Why do 80% of bovine and human oocytes fail to develop into embryos during *in vitro* fertilization and culture procedures? Why, in the last 25 years, have we been unsuccessful in improving the proportion of transferable embryos collected from superovulated donor cows? The fundamental answer to these questions relates to ***oocyte competence***. One oocyte, or egg, resides in each ovarian follicle. The ovary contains thousands of follicles yet fewer than 1% ovulate (release the contained oocyte for fertilization) during a reproductive lifetime – the remaining 99% are *genetic waste*. Although we are capable of recovering the genetic material through superovulation, in vitro fertilization (IVF), and gamete recovery and follicular transfer (GRAFT), we have not addressed the high rate of loss inherent with these techniques. The reason for such *inherent* loss is unknown.

We have become extremely proficient at controlling follicle development and ovulation, and are one of perhaps only three centers in the world that have successfully developed the fixed-time insemination technique in cattle - a technique that permits the producer to pre-schedule the breeding season to fit labor and management resources. We have achieved astounding results in

the last 2 years; i.e., pregnancy rate equivalent to that of natural breeding. Our success is a direct result of previous detailed studies of follicle dynamics and its control. We are, however, still left with *inherent loss* that limits pregnancy rate. Further, we do not have an adequate understanding of the effects of common fertility treatments on oocyte competence for any species – including humans.

Reproductive efficiency is the single most important factor influencing the profitability of livestock production. Livestock production in Saskatchewan generates $ 1.5 billion annually in farm cash receipts. We cannot yet predict the level of improvement in reproductive efficiency which may result from the proposed studies, but we do know that the potential for improvement is substantial (40% over natural breeding and 80% for *in vitro* embryo production). Improvement as modest as 1% represents an economic return of $15 million. We anticipate that unraveling the mysteries of oocyte competence will result in economic returns far greater than 1%.

Ovarian follicular and luteal dynamics in cattle have been extensively studied using ultrasonography in our laboratory. We have shown that ovarian follicles grow in a wave-like pattern and each "follicular wave" is preceded by a surge of follicle stimulating hormone (FSH). This is followed by growth of a cohort of follicles and within 3 days, selection of a dominant follicle while subordinate follicles undergo atresia and die. The selection of the dominant follicle involves a decrease in circulating FSH concentrations and the establishment of receptors for luteinizing hormone (LH). The decline in FSH results in alterations in FSH-dependent growth factors and hormones within the group of growing follicles which may have important effects on the developmental competence of oocytes in those follicles. Progesterone is also part of a negative feedback mechanism that controls LH secretion in cattle. Low levels of progesterone modulate changes in LH secretion resulting in the development of persistent dominant follicles and low pregnancy rates when the animal comes into heat. Consequently, progesterone-based protocols for the control of estrous cycle in cattle may be expected to have a significant effect on the developmental capacity of oocytes. We have demonstrated the effects of follicular wave status on superovulatory response to exogenous FSH and in the synchrony of estrus and ovulation in fixed-time AI programs. Our studies on the manipulation of the ovarian function have shown that hormones (progesterone, estradiol, LH or GnRH,), or follicular ablation can be used to synchronize follicular wave emergence. Ablation of a dominant follicle utilizing ultrasound-guided follicular ablation results in synchronous emergence of a new follicular wave 1 to 2 days later, while treatment of progestin-treated animals with estradiol-17β results in the emergence of a new follicular wave in approximately 4 days. Treatment with GnRH or LH during the first follicular wave was partially effective in inducing ovulation and synchronous emergence of a follicular 2 days later. The combination of a progesterone-impregnated intravaginal device (CIDR-B) with a method of follicular wave synchronization (estradiol-17β and progesterone, GnRH/pLH or follicular ablation) in an estrus synchronization program generated new alternatives for fixed-time AI with high fertility; e.g., the use of a CIDR-B device in a GnRH or pLH-based ovulation synchronization program resulted in a 30% increase in pregnancy rates after a fixed-timed AI in heifers. We have also performed preliminary experiments suggesting that oocytes from follicles in early stages of atresia are more capable of forming embryos. Initial studies in our laboratory indicate that oocytes obtained from subordinate follicles during the late-static or early-regressing phase, even in the absence of an endogenous LH surge, have a higher rate of development to the blastocyst stage

than those obtained during the growing phase. However, intrinsic factors affecting the developmental capability of oocytes to undergo fertilization and subsequent embryo development remain to be determined.

This research was designed to investigate hormonal mechanisms that affect the developmental competence of oocytes (eggs) in the cow using an *in vitro* (in the test tube) embryo production system. Specifically, we investigated the relationship between the stage of follicle development and the potential of the oocyte to develop into an embryo, and the influence of hormonal treatments commonly used for synchronization and superstimulation on the developmental capacity of oocytes in the ovary. We were also interested to know how treatments that result in the development of persistent follicles affect the developmental competence of oocytes in those follicles. Initial experiments were designed to examine the fundamental relationships between follicle maturation and oocyte competence, while later experiments were designed to examine the influences of applied synchronization and stimulation regimens on oocyte competence.

(c) The research results of this project are presented under 3 main experimental studies (as originally proposed) based on factors expected to influence oocyte competence: 1) Follicular status, 2) Progestational status, and 3) Gonadotropin status (LH & FSH). Rationale, Methods and Results are presented separately for each of these studies.

STUDY 1. EFFECT OF THE PHASE OF FOLLICULAR DEVELOPMENT ON OOCYTE COMPETENCE.

In cattle, follicle dimension has been used as the main criterion for selection of oocytes for in vitro embryo production. However, follicles with similar diameters may be in very different physiologic phases. Synchronization and superstimulation regimens alter the growing phases of dominant and subordinate follicles by either inducing or delaying regression and by preventing dominant follicle selection, respectively. This effect may be expected to influence the developmental competence of oocytes in those follicles. Data from an initial study in our laboratory suggest that oocytes from subordinate follicles in the late static or early regressing phase have greater competence to develop *in vitro* than those of growing-phase subordinate follicles. However, the specific relationship between the growing phase of follicle development and oocyte competence has not been critically investigated.

The aim of Experiment 1 was to investigate whether morphology and developmental competence of cumulus oocyte complexes (COC) are related to the: 1) phase of development of the follicle, and 2) presence of the CL and/or the dominant follicle in the ovary from which the COC were collected. Cows (n = 143) were given a luteolytic dose of $PGF_{2\alpha}$ and, 8 days later, underwent transvaginal ultrasound-guided ablation of follicles ≥4 mm to induce emergence of a new follicular wave. Cows (n = 10 to 20 per replicate) were slaughtered on Days 2, 3, 5 or 7 (Day 0 = follicular wave emergence), equivalent to the growing, early static, late static, and regressing phases of subordinate follicle development. COC were collected from subordinate follicles ≥3 mm. COC were classified as denuded, degenerated, or healthy, and underwent IVM-IVF-IVC. The proportion of oocytes that developed to the blastocyst stage was higher ($P < 0.05$) in those collected on Day 5 after wave emergence (23%) than on Days 2 (12%), 3 (13%) or 7 (16%). Data did not support the hypothesis of a local effect of the CL or dominant follicle. We conclude that a positive relationship exists between early follicular regression and oocyte competence.

Moreover, morphologic characteristics of oocyte quality used in this study were not predictive in identifying competent oocytes. *(see Vassena et al., 2003a)*

There is a need to identify reliable indicators of oocyte competence and develop a simple, noninvasive method to assess oocyte competence. The aim of Experiment 2 was to investigate the relationships among ultrasonographic attributes of a follicle, its stage of development and the competence of the oocyte that it contains. We tested the hypotheses that follicular echotexture characteristics are related to the: 1) phase of development of the follicle, 2) presence of the corpus luteum (CL) and/or the dominant follicle in the ovary, and 3) developmental competence of cumulus oocyte complexes (COC) from the same ovary. Crossbred beef cows (n = 143), age 4 to 14 years, were given a luteolytic dose of dinoprost to cause ovulation. Ultrasound-guided ablation of all follicles $\geq$ 4 mm was done 8 days later to induce new follicular wave emergence during a luteal phase. Ultrasonographic images of dominant follicles and the 3 largest subordinate follicles (n = 402 follicles; 84 cows) were acquired on Days 2, 3, 5 or 7 of the follicular wave (Day 0 = wave emergence), i.e. growing, early static, late static, and regressing phases of subordinate follicle development, respectively. From a subset of these animals (n=33), ovaries were collected within 30 minutes of slaughter and COC from subordinate follicles $\geq$ 3 mm underwent in vitro maturation, fertilization and culture to the blastocyst stage. Image analysis revealed differences in echotexture between dominant and subordinate follicles among Days 2 to 7 of the follicular wave. Images of dominant and subordinate follicles at Day 7 of the wave displayed consistently lower grey-scale values ($P < 0.05$) in the peripheral antrum, follicular wall and perifollicular stroma than all other days. Follicle images displayed a consistent pattern of variation in echotexture among follicular phases. Data did not support the hypothesis of a local effect of the CL or dominant follicle on follicular echotexture. Echotexture values of the perifollicular stroma were lower in ovaries that did not produce embryos compared to ovaries that produced embryos. Our results showed that changes in follicular image attributes are consistent with changes in follicular status. The sensitivity of the technique is not yet sufficient for use in a diagnostic setting, but results provide rationale for further development of image analysis as a tool for evaluating oocyte competence in situ. (see *Vassena et al. 2003b)*

Study 2. Effect of Progesterone on Oocyte Competence

Less than half of natural follicular waves are ovulatory. Only waves that produce a dominant follicle during the progesterone decline (luteolysis) are ovulatory. This study was designed to investigate the effect of progesterone on oocyte competence obtained at otherwise comparable follicular phases so as to determine if existing hormonal regimens have any influence on the ability of oocytes to produce a calf.

Results of past studies have shown that prolonged treatment with progestagens for estrus synchronization results in poor fertility. The specific objectives of this experiment were to compare the developmental capacity of oocytes that develop 1) under low- versus high-progesterone concentrations (to mimic ovulatory and anovulatory waves), and 2) from persistent over-dominant follicles. These objectives were addressed in a single experiment rather than 2 experiments, as originally proposed. Hereford crossbred heifers with a functional corpus luteum (CL) were assigned randomly to three groups (n=10/group) intended to provide low-, prolonged-low- and high- circulating progesterone (P4). Follicular wave emergence was synchronized

among heifers by follicular ablation, and low-P4 implants (once-used CIDR) were placed intravaginally. Heifers in low-P4 and prolonged-low-P4 groups were treated with 500μg PGF2α analog 12h apart on the day of ablation while heifers in high-P4 group were permitted to retain their CL. Beginning 24h post-ablation, heifers in all groups were treated i.m. with 200mg FSH divided bid over 4 days (Day 0=first FSH treatment). Heifers in high-P4 group were given PGF2α on Day 3. The CIDR devices were removed from heifers in low-P4 and high-P4 groups on Day 3, and from prolonged-low-P4 group on Day 8. All heifers were treated i.m. with 25mg LH 24h after CIDR removal. Heifers were artificially inseminated at 24h and 36h post-LH treatment and were sent to slaughter 3 days after the last insemination. The embryos/ova were flushed from the uterine tubes. Ovarian ultrasonography was conducted on Days -1, 4, and 24h prior to slaughter. Data were analyzed using one-way ANOVA and Chi-squared tests. Plasma LH pulse frequency (36h before CIDR removal) was lowest and Day 3 P4 concentration was highest in high-P4 group ($P<0.01$). The number of follicles ≥12mm was highest ($P=0.02$) and the number of follicles 9 to11 mm tended to be higher ($P=0.07$) in prolonged-low-P4 group. No ovulations were detected in prolonged-low-P4 group. The embryo/ovum recovery rate was higher ($P<0.01$) in high-P4 (139/204, 68%) versus low-P4 (109/218, 50%) group, but the proportion of fertilized ova was higher ($P<0.01$) in low-P4 (94%) versus high-P4 group (73%). It was concluded that in FSH treated heifers, oocytes that developed under a high-progesterone milieu were less capable of developing into embryos, whereas oocytes that developed under a prolonged low- progesterone milieu failed to be ovulated. *(see Jaiswal et al. 2006a)*

Study 3. Influence of LH on Oocyte Competence

Oocytes of dominant follicles undergo morphological alterations as a result of the preovulatory LH surge. The alterations are thought to be necessary for final maturation and competence to become fertilized and develop into a viable embryo. Superstimulation and synchronization schemes incorporate the use of LH to induce or mimic the conditions of ovulation, but the effects of these treatments on oocyte competence are unknown. Moreover, data from a number of studies in our lab and others, indirectly suggest that the process of follicular atresia induces oocyte maturation similar to a preovulatory LH surge. However, this observation has not been tested directly. Furthermore, the interval between the end of superstimulatory treatment and oocyte collection appears to affect oocyte competence, but the mechanism is unknown. Study 3 was designed to answer the fundamental question "is it possible to induce optimal oocyte development by controlling the phase of follicle development and exposure to LH?" From a practical point of view, this would mean more efficient and simpler protocols for *in vitro* embryo production.

The objectives of initial experiments were to distinguish between the effects of LH and FSH on nuclear maturation of oocytes. In Experiment 1, the effects of duration of purified bovine FSH (i.e., no LH activity) treatment for superstimulation and time interval thereafter on nuclear maturation of oocytes from large (≥9 mm) and small (≤8 mm) follicles was examined in cows with basal levels of LH (i.e., high endogenous progesterone). Follicular wave emergence was synchronized in cows by ablation of follicles ≥ 5 mm on Day 5 to 7 (Day 0=ovulation). A functional corpus luteum was maintained throughout. An equivalent total dose of purified bovine FSH was given over 3 days (25 NIH-S1 units every 12 hours ; n=10) or over 4.5 days (16.7 NIH S1 units every 12 hours; n=10) starting at the expected time of wave emergence. Cumulus oocyte

complexes (COC) were collected by transvaginal ultrasound-guided needle aspiration at 12, 48, 84, and 120 hours after the last FSH injection. COCs were classified and oocytes were fixed and stained with orcein stain. Nuclear status of oocytes was classified as immature (germinal vesicle stage), intermediate (germinal vesicle breakdown to metaphase I) and mature (metaphase II). Data were analyzed using Fisher's exact test. Overall, 51.2% (173/338) oocytes displayed GVBD or more advanced stages of nuclear maturation. There were no differences in the proportion of immature oocytes ($P>0.05$) between 12, 84 and 120 hours post FSH treatment. There were more intermediate oocytes at 12 and 120 hours in the 6-dose group than the 9-dose group (47.5% vs. 14.3% and 56.8 vs. 34.8%, respectively; $P<0.05$). More ($P<0.05$) oocytes with heterogeneous cytoplasm were recorded at 120 hours in the 9-dose group than all other time periods. We concluded that in the absence of elevations in LH, FSH alone could initiate nuclear maturation of oocytes in vivo. The duration of FSH treatment did not affect the nuclear status of oocytes; however, FSH starvation (in the absence of a preovulatory LH surge) did not trigger completion of meiosis I (no oocytes reached Metaphase II). *(see Saravanan et al, 2004)*

Using a similar design, Experiment 2 was done as a follow-up to distinguish the effects of LH and FSH on the developmental competence of bovine oocytes in vitro. A follicular wave was induced during the mid-luteal phase in Hereford-cross heifers (n=40) by ablation of all follicles ≥ 5 mm in diameter. Heifers were given a total dose of 150 IU of purified bovine FSH (NIH S1 units) im, divided bid for 3 days (i.e. 6 treatments of 25 IU each). Cumulus-oocyte-complexes (COC) were collected by transvaginal ultrasound-guided follicle aspiration at 12, 48, 84 and 120 hours after the last FSH injection (n = 10/group). The COC were matured and fertilized in vitro, and embryos were cultured in vitro for 7 days. Blastocyst development was compared among groups by Fisher Exact test. A total of 976 follicles were aspirated and 550 COC were obtained from 40 heifers. The proportion of oocytes that developed into blastocysts tended to be greater ($P<0.1$) in the 48 hr- (13%) and 84 hr-groups (16%) than in the 12 hr- (8%) and 120 hr-groups (8%). A greater proportion ($P<0.05$) of 8- and 16-cell embryos was obtained from the 120 hr-group (25%) than in the 12 hr- (12%) and 84 hr-groups (14%). We concluded that in a low LH mileau, FSH starvation influences oocyte competence; the optimum time for oocyte collection for in vitro fertilization and culture was 48 to 84 hours after the last FSH treatment. *(see Saravanan et al, 2005a)*

The objectives of the final experiments in this series were to determine the role of LH in meiotic maturation of oocytes (ability to complete first meiotic division) in vivo, and to test the hypothesis that aging is associated with perturbations in oocyte meiotic maturation. In Experiment 3, superstimulatory treatment with FSH was initiated in mother-daughter pairs (14-15yr and 3-5yr respectively, n=7 pairs) after follicle wave synchronization. A functional CL was maintained throughout the experiment. Follicles were aspirated from one ovary 5 days after wave emergence. LH was given immediately thereafter, and follicles of the other ovary were aspirated 22hr later. The meiotic stage of oocytes was evaluated by light microscopy after aceto-orcein staining. In Experiment 4, 14-17yr old cows (n=10) and their 3-7yr old daughters (n=12) were superstimulated with FSH as in Experiment 3. Luteolysis was induced with PGF2α 3.5 days after wave emergence, and LH was given 32 hr later. Follicles were aspirated 18-20hr after LH treatment. Expanded cumulus-oocyte complexes (COC) were matured in vitro for 6 hr whereas compact COC were matured in vitro for 24 hr. The meiotic stage was evaluated after Giemsa staining of chromosomal spreads. Data were compared by Student t-test and Chi-Square test. The

lower number of follicles (mean±SEM) ≥6mm on the day of oocyte retrieval in old versus young cows did not reach significance in Experiment 3 (27±6 versus 38±3; P=0.12), but did in Experiment 4 (30±3 versus 47±5; P=0.01). Concentrations of LH and progesterone did not differ (P>0.4) between age groups in either experiment. In Experiment 3, most of the oocytes recovered from old and young cows before (48/52 versus 71/75; P=0.6) or after (58/61 versus 61/66; P=0.5) LH exposure had not reached metaphase I (i.e., germinal vesicle or germinal vesicle breakdown stage, but no metaphase plate). In Experiment 4, old cows had higher (P=0.02) numbers of expanded COC at collection than their young daughters (68/182 versus 95/350), and a tendency for a higher number (P=0.08) of metaphase II oocytes after 6 hrs of in vitro incubation (69/115 versus 101/203). Luteolysis prior to LH treatment (Experiment 4) resulted in a greater proportion of metaphase II oocytes (170/318 versus 2/66; P<0.01) than LH treatment during a high progesterone phase (Experiment 3). We concluded that a period of elevated LH pulse frequency (i.e., after luteolysis) before LH treatment (mimicking the preovulatory surge) was a necessary stimulus for in vivo COC expansion and oocyte meiotic maturation. The hypothesis that reproductive senescence is associated with perturbation in oocyte meiotic maturation was not supported.

(d) **Conclusions and Recommendations**

1. A positive relationship exists between early follicular atresia and oocyte competence. Moreover, the classical method of morphologic assessment of oocyte quality, originally published over 25 years ago, was not predictive in identifying competent oocytes. Finally, the presence of the corpus luteum or dominant follicle had no local effect on the developmental competence and morphology of the cumulus oocyte complexes (COC).

2. Follicular echotexture characteristics were related to the phase of development of the follicle, and the developmental competence of the COC. The sensitivity of the technique, however, is not yet sufficient for use in a diagnostic setting, but results provide rationale for further development of image analysis as a tool for evaluating oocyte competence.

3. In cattle given superstimulatory treatment, oocytes that developed under a high-progesterone milieu were less capable of developing into embryos, whereas oocytes that developed under a prolonged low- progesterone milieu failed to be ovulated. This finding is expected to have profound implications on present-day superovulatory protocols and fixed-time AI protocols. This is an exciting area that we will continue to investigate in our next ADF grant period.

4. In the absence of elevations in LH, FSH alone initiated the process of nuclear maturation of oocytes in vivo. The duration of FSH treatment did not affect the nuclear status of oocytes, but contrary to expectation and results of others, FSH starvation (in the absence of a preovulatory LH surge) did not trigger completion of meiosis I (i.e., no oocytes reached Metaphase II).

5. In studies to distinguish between the effects of LH and FSH on oocyte competence, we concluded that FSH starvation in a low LH milieu influences oocyte competence. The optimum time for oocyte collection for in vitro fertilization and culture is 48 to 84 hours after the last FSH treatment.

6. A period of elevated LH pulse frequency (mimicking proestrus) before LH treatment (mimicking the preovulatory surge) provides a necessary stimulus for in vivo COC expansion and oocyte meiotic maturation. Reproductive aging was not associated with perturbation in oocyte meiotic maturation.

(e) **Acknowledgements** – The Saskatchewan Agriculture Development Fund was acknowledged in all manuscripts, abstracts and conference platform and poster publications.

(f) **Publications related to this project:**

Jaiswal RS, Singh J, Nagra HS, Grafton T, Ratto MH, Malhi PS and Adams GP. (2006a) Effect of progesterone on oocyte competence after superstimulation in beef heifers. Theriogenology (submitted)

Jaiswal RS, Singh J, Nagra HS, Grafton T, Ratto MH, Malhi PS and Adams GP. (2006b) Oocyte competence under different progestational environments. 2nd Annual Reproductive Science and Medicine Research Symposium. February 24, 2006, p 4.

Malhi P, Adams GP, Singh J. (2006) The bovine model for reproductive aging: Superovulatory response. Proceedings of the annual conference of the International Embryo Transfer Society, Reproduction Fertility and Development 18: 290.

Malhi P, Adams GP, Mapletoft RJ, Singh J. (2005) Bovine model to study age-associated infertility in women: Developmental competence of oocytes. Theriogenology 64: 778.

Malhi PS, Adams GP, Mapletoft RJ, Singh J (2004) Effect of maternal age on gonadotropin secretion and oocyte competence in a bovine model. Proceedings of the annual meeting of the Society for the Study of Reproduction, Vancouver, BC August 2004 Abstract 35.

Malhi PS, Adams GP, Ratto MH, Nagra HS, Singh J. (2006) Oocyte meiotic maturation in a bovine model of reproductive aging. 2nd Annual Reproductive Science and Medicine Research Symposium. February 24, 2006, p 5.

Saravanan D, Mayes M, Adams GP, Singh J. (2005a) Effect of FSH starvation on the developmental competence of bovine oocytes in vitro. Proceedings of the annual meeting of the Society for the Study of Reproduction, Quebec City, Quebec, July 24-27, abstract M670.

Saravanan D, Mayes MA, Adams GP, Singh J (2005b) Effect of FSH starvation on competence of bovine oocytes in vitro. The 12th Annual Life and Health Sciences Student Research Conference, University of Saskatchewan, Mar. 13 and 14, 2005, p 82.

Saravanan D, Adams GP, Malhi PS, Mayes MA, Ratto MH, Singh J (2004) Effect of FSH on nuclear maturation of oocytes in the bovine model. Proceedings of the 50th Annual Conference of the Canadian Fertility and Andrology Society, Nov. 24-27 2004, p 63.

Saravanan D, Adams GP, Malhi P, Mayes MM, Ratto MH, Singh J (2004) Effect of FSH on nuclear maturation of bovine oocytes in vivo. 11th Annual Life Sciences Student Research Conference University of Saskatchewan Saskatoon Canada. May 13-14, 2004 (page # 108).

Singh, J., Adams GP, Pierson, R.A. (2003) Promise of new technologies for visualizing ovarian structure and function in domestic animals. Animal Reproduction Science 78:371-399.

Singh, J., Domínguez, M., Jaiswal, R., Adams GP (2004) A simple ultrasound test to predict the superstimulatory response in cattle. Theriogenology 62:227-243.

Vassena, R., Mapletoft, R.J., Allodi, S., Singh, J., Adams GP (2003a) Effect of follicular stage on the morphology and developmental competence of the cumulus-oocyte-complex in cattle. Theriogenology 60:923-932.

Vassena, R., Adams GP, Mapletoft, R.J., Pierson, R.A., Singh, J. (2003b) Ultrasound image characteristics of the ovarian follicle in relation to developmental competence of the oocyte in cattle. Animal Reproduction Science 76:25-41.

(g) **Literature Cited**
(see reference lists in publications)

(h) **Appendices**
(see posters and platform presentations appended)

(i) **Other**
(copies of publications enclosed)

Appendices

Competence of oocytes developing under different progesterone environments

Jaiswal RS, Singh J, Nagra HS, Grafton T, Ratto MH, Malhi PS and Adams GP

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Introduction

- Synchronized breeding program
  - Treatment should minimize labor & animal handling
  - Rapid and synchronized decrease of circulating progesterone
  - Synchronized growth & ovulation of dominant follicle

Schemes for estrus synchronization

- Long-term progesterone (>14 days)
  - Good estrus synchronization
  - Development of over-sized follicle
  - Poor fertility
- PGF2α (11 or 14 days apart)
  - Response depends on the status of follicle at the time of treatment
- Short-term progesterone (7 to 10 days+ PGF2α)
  - Reasonable synchronization of estrus & ovulation

Questions to be answered...

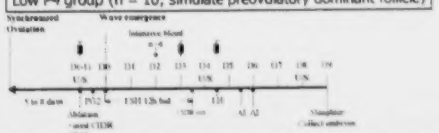
- Difference in pregnancy rates: 2- versus 3-wave cycles (Ahmed et al., 1997; Townson et al., 2002; Bleach et al., 2004; Celik et al., 2005).
- Oocytes from prolonged dominant follicle undergo premature maturation in vivo (Mihm et al., 1994; Revah and Butler, 1996).

Objectives

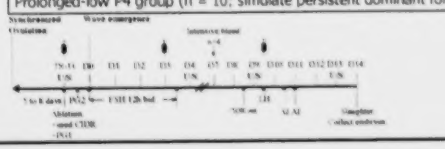
- To compare the developmental capacity of oocytes that develop
  - 1) under low- versus high-progesterone concentrations, and
  - 2) in persistent over-sized dominant follicles.

Materials & Methods

Low P4 group (n = 10; simulate preovulatory dominant follicle)

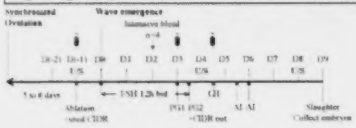


Prolonged-low P4 group (n = 10; simulate persistent dominant follicle)



Materials & Methods (contd.)

High P4 group (n = 10; simulate luteal phase dominant follicle)



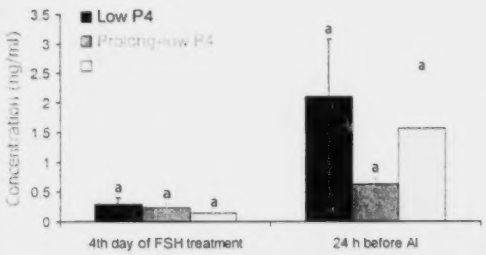
- Hormone assays: LH and Progesterone
- Data analysis: ANOVA & Chi-squared test

Profile of LH pulse

LH profile (ng/ml)	Low P4	Prolonged -low P4	High P4
Basal LH concentration	0.0 ± 0.01[a]	0.0 ± 0.00[ab]	0.0 ± 0.00[b]
LH pulse amplitude	0.2±0.05[a]	0.2±0.02[a]	0.3±0.11[a]
LH pulse frequency	6.0±0.00[a]	5.3±0.85[a]	1.8±0.48[b]

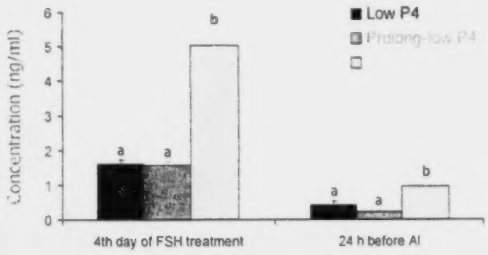
[ab]values (mean±SEM) in a row with no common superscripts are different (P < 0.05)

Plasma LH concentrations



[ab]within a day, means (±SEM) with different superscripts differed (P < 0.05)

Plasma P4 concentrations



[ab]within a day, means (±SEM) with different superscripts differed (P < 0.05)

Follicle numbers before ovulation

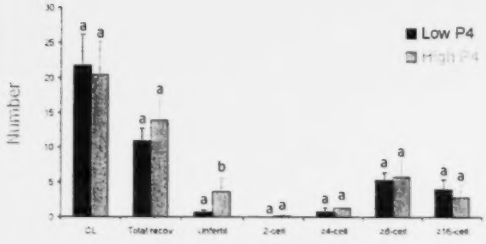
Follicle category	Low P4	Prolonged-low P4	High P4
≤5 mm	11.3 ± 2.55[a]	7.3 ± 1.68[a]	8.9 ± 1.80[a]
6 to 8 mm	18.7±6.48[a]	13.2±4.43[a]	17.4±5.46[a]
9 to 11 mm	7.6±2.50[ab]	11.2±2.33[a]	4.2±0.98[b]
≥12 mm	1.8±0.63[ab]	3.9±1.32[a]	0.2±0.13[b]
Total follicles	39.4±9.99[a]	35.6±5.23[a]	30.7±6.99[a]

[ab]values (mean±SEM) in a row with no common superscripts are different (P < 0.05)

Results

- Follicles in the prolonged-low progesterone group failed to ovulate.

Ovulation and embryo collection



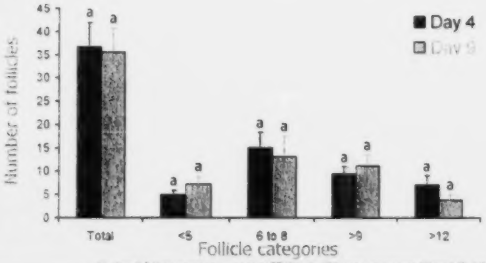
*within a category, means (±SEM) with different superscripts differed (P < 0.05)

Ova/embryos in low versus high P4

	Low P4	High P4
Ovum/embryo recovery rate	109/218 (50%)[a]	139/204 (68%)[b]
Proportion of fertilized ova	102/109 (94%)[a]	102/139 (73%)[b]

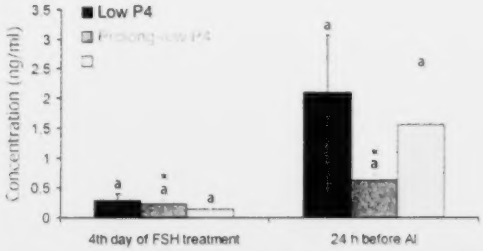
*values (mean±SEM) in a row with no common superscripts are different (P < 0.05)

Prolonged-low P4 group



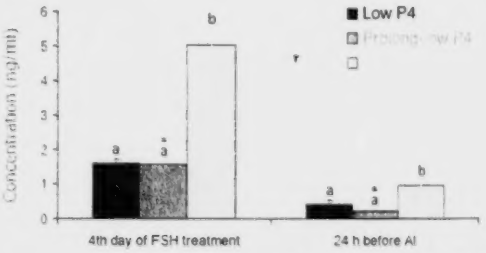
*within a follicle category, means (±SEM) with different superscripts differed (P < 0.05)

Plasma LH concentrations



*within a day, means (±SEM) with different superscripts differed (P < 0.05)

Plasma P4 concentrations



*within a day, means (±SEM) with different superscripts differed (P < 0.05)

Conclusions

- In FSH treated heifers
  - Oocytes that develop under a high progesterone milieu are less capable of developing into embryos
  - Oocytes that develop under a prolonged-low progesterone milieu fail to be ovulated

Why?

Acknowledgements

- Funding Support from:
  - ADF Saskatchewan
  - NSERC
- Goodale Cattle Research Farm for maintaining animals

The Bovine Model for Reproductive Aging: Superovulatory Response

Pritpal S. Malhi, Gregg P. Adams, Reuben J. Mapletoft and Jaswant Singh
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WCVM

INTRODUCTION

- Reproductive aging in women and cows is associated with elevated circulating concentrations of FSH *(Klein et al 1996, Malhi et al 2005)*.
- Maternal age is associated with a decline in fertility and a lower superstimulatory response in humans *(Klein et al 2001, Dew et al 1998)*.
- There is a need for a well characterized animal model for testing age-associated changes in ovarian function in humans.
- The bovine model is well characterized and has relevance to ovarian function in women (Adams and Pierson 1995, Baerwald et al 2003, Malhi et al 2005)
- The average life expectancy of cattle is 19 years; but 50% of the herd is infertile by 13 years of age *(Erickson et al 1976)*.

OBJECTIVE

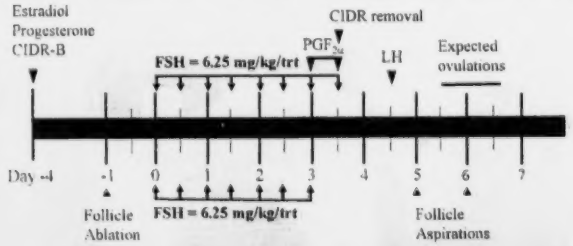
To investigate the effect of reproductive aging in the bovine model on
1) recruitment of small antral follicles (2-5 mm) in an induced follicular wave.
2) follicular and ovulatory response following gonadotropin treatment.

HYPOTHESES

Aging in cattle is associated with
1) fewer follicles recruited into an ovarian follicular wave.
2) a reduced ovarian follicular and ovulatory response after treatment.

MATERIAL AND METHODS

EXPERIMENT 1 **Old cows 13-16 yr:** n = 6 in year 1 and n = 9 in year 2
Young daughters 3-6 yr old: n = 8 in year 1 and n = 9 in year 2



EXPERIMENT 2 **Old cows 13-16 yr:** n = 7 in year 1 and n = 9 in year 2
Young daughters 3-6 yr old: n = 7 in year 1 and n = 9 in year 2

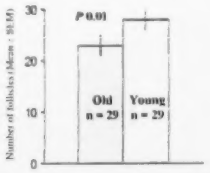
- ❖ Daily ultrasound examinations in Experiments 1 and 2
- ❖ A total of 31 and 33 superstimulations in old and young cows, respectively
- ❖ Statistical analyses: Paired t-test and Pearson's correlation coefficient

RESULTS

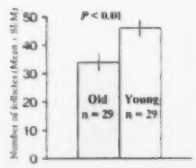
- ❖ Old and young cows did not differ ($P > 0.3$) in mean body weight or dose of FSH treatment.

EXPERIMENTS 1 and 2

A) Follicles (2-5 mm) at wave emergence



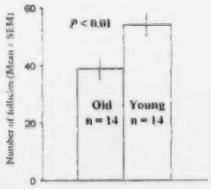
B) Follicles (≥6 mm) at Day 5 of treatment



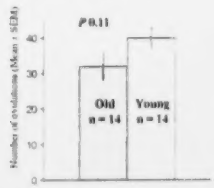
- ❖ The number of follicles ≥ 6 mm in successive superstimulatory cycles was correlated within animals ($r > 0.8$; $P < 0.0001$)

EXPERIMENT 1

A) Follicles (≥6 mm) at Day 5 of treatment



B) Superovulatory response



- ❖ The number of ovulations in successive superstimulatory cycles was correlated within animals ($r = 0.6$; $P = 0.04$)

CONCLUSIONS

- Hypothesis 1 supported:
  - reduced number of small follicles (2-5 mm) at follicular wave emergence.
- Hypothesis 2 partially supported:
  - reduced number of large follicles (≥ 6 mm) after gonadotropin treatment.
  - Superstimulatory response tended to be lower in old cows.
- The follicular and ovulatory response was repeatable within individuals.

Supported by grants from the *Natural Sciences and Engineering Research Council of Canada* and *Saskatchewan Agriculture Development Fund*
We gratefully acknowledge Biomiche Animal Health Canada Inc for providing FSH (Folltropin-V), LH (Lutropin-V) and Intra-vaginal progesterone releasing device (CIDR-B) for the study.

IETS 2006, Orlando USA
Poster # 366 Abstract # 13553

Bovine Model to Study Age-Associated Infertility in Women: Developmental Competence of Oocytes

Pritpal S. Malhi[1], Gregg P. Adams[2], Reuben J. Mapletoft [2] and Jaswant Singh[1]

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Western College of Veterinary Medicine
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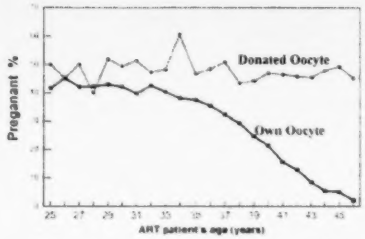
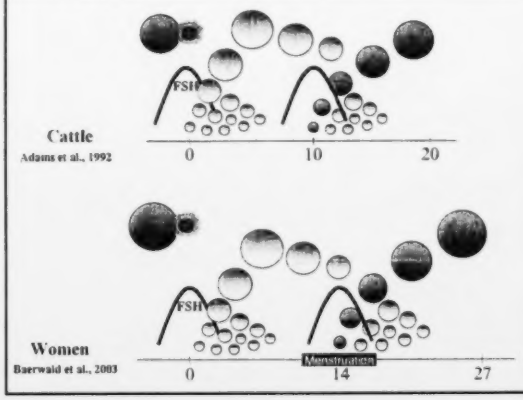
Oocyte Donation

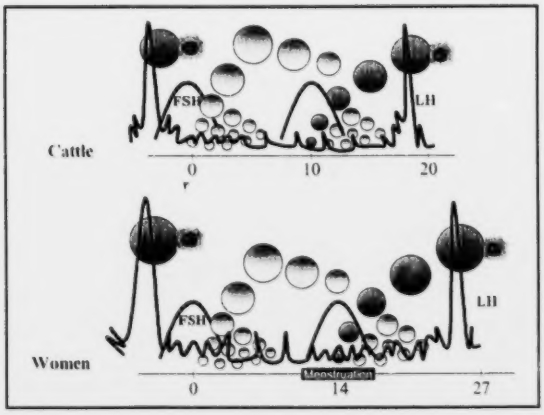


2001 Assisted Reproductive Technology Success Rates, National Summary and Fertility Clinic Reports
http://www.cdc.gov/reproductivehealth/art.htm

Issues and Challenges

- Increased chromosomal abnormalities
- Prediction of fertility
- Ethical constraints
- Lack of a well-characterized animal model





Aging in Cattle

- Rise in circulating concentrations of FSH
- Tendency for lower plasma progesterone in luteal phase
- Fewer 4-5 mm follicles at follicular wave emergence

Malhi et al., 2005 Biology of Reproduction

Objective

To study the effect of age on oocyte developmental competence in cattle

Hypothesis

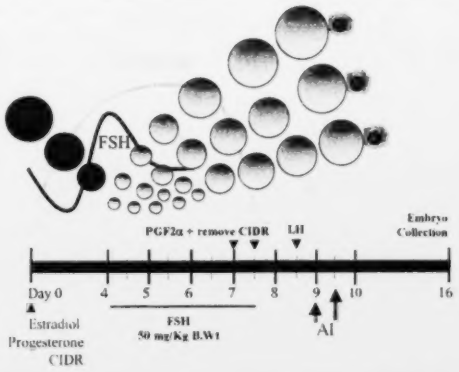
Aging in cattle is associated with reduced developmental competence

Methods

Beef cows (N=20)

Old cows (n=10)	Daughters (n=10)

Year 1	**13-15 years, n = 6**	**3-5 years, n = 8**
Year 2	**14-16 years, n = 9**	**4-6 years, n = 9**



Embryo Transfers

Year 1

Single embryo transferred (n = 4 recipients, 2-5 years)

2 embryos transferred (n = 28 recipients, 2-5 years)

Year 2

Single embryo transferred (n = 41 recipients, 2-5 years)

2 embryos transferred (n = 26 recipients, 2-5 years)

Statistical Analysis

- Single point data compared by Student's t-test
  - Number of ovulations
  - Number of ova/embryos
- Proportional data compared by Fisher's exact test
  - Embryos
  - Unfertilized or uncleaved zygotes
  - IETS grade 1-2 transferable embryos
  - Embryo survival

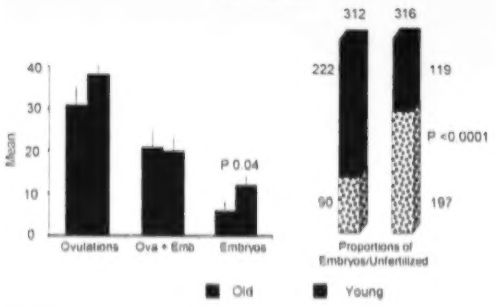
Results (Year 1)

	Old Cows (n=6)	Young cows (n=8)	*P* Value
Mean ovulations	33 ± 5	34 ± 2	0.4
Mean Ova + embryos	22 ± 5	17 ± 4	0.2
Mean embryos	4 ± 2	9 ± 3	0.2
Proportion of embryos	23 (17%)	73 (54%)	<0.0001
Transferable embryos	16 (70%)	48 (66%)	0.8
Proportion of unfertilized	110 (83%)	63 (46%)	<0.0001
Embryos transferred	14	46	
Calves born	6 (43%)	20 (42%)	0.8

Results (Year 2)

	Old Cows (n=9)	Young cows (n=8)	*P* Value
Mean ovulations	32 ± 6	39 ± 4	0.3
Mean Ova + embryos	20 ± 5	23 ± 3	0.7
Mean embryos	7 ± 3	16 ± 4	0.08
Proportion of embryos	67 (37%)	124 (69%)	<0.0001
Transferable embryos	43 (64%)	85 (69%)	0.63
Proportion of unfertilized	112 (63%)	56 (31%)	<0.0001
Embryos transferred	43	50	
Calves born	12 (28%)	22 (44%)	0.13

Results



Conclusions

- Hypothesis supported: reduced oocyte competence (fewer embryos) in old cows
- Intrinsic defect in oocyte or defect in gamete transport
- Bovine model useful for studying oocyte-associated infertility

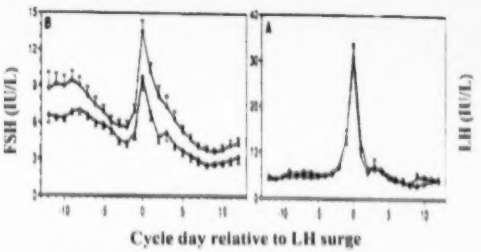
Acknowledgements

- Drs Adams, Mapletoft and Singh
- Natural Sciences and Engineering Research Council of Canada and ADF Saskatchewan
- University of Saskatchewan
- Society for Theriogenology

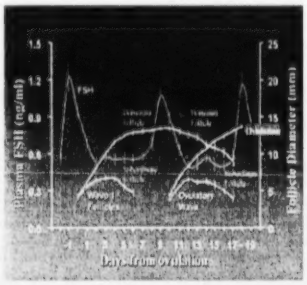
Effect of Maternal Age on Gonadotropin Secretion and Oocyte Competence in A Bovine Model

Western College of Veterinary Medicine
University of Saskatchewan, Canada

Infertility



Bovine Model



- Follicular waves
- Follicular selection
- Monovular
- Finite number follicles
- Life expectancy: 19 years

Ericson et al., 1976 BOR

Objective

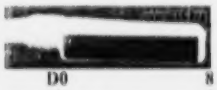
To develop and validate a bovine model for the study of reproductive aging in women

Hypothesis

Maternal age is associated with altered gonadotropin secretion and reduced oocyte competence in cattle

Experiment 1

Aged cows, 13-15 years: n=10 **Daughters, 1-4 years: n=9**



Plasma Samples – every 12 hrs

D0 8 18 …D0

Interovulatory Interval (IOI)

Ovulation Synchronization

every 15 min plasma samples for 8 hrs (n=9 per group)

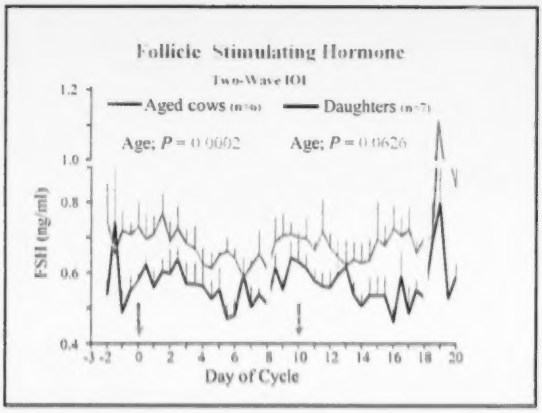
Results

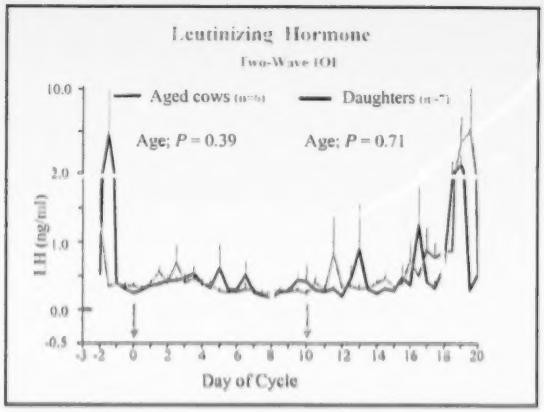
- The length of IOI

  21.2 ± 0.7 days in aged vs. 20.9 ± 0.6 days in young, $P > 0.7$

- Proportions of 2-Wave IOI

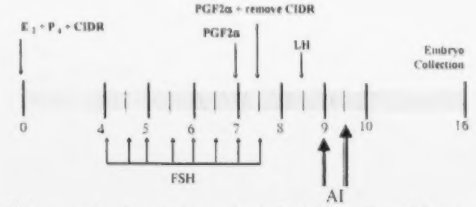
  6/10 in aged cows vs. 7/9 in young cows, $P = 0.6$





Experiment 2

Aged cows, 13-15 years: n=6 **Daughters, 3-5 years:** n=8



Embryos transferred as pairs into synchronized recipients (n=32, age: 2-5years)

Results

	Aged Cows (n=6)	Young cows (n=8)	*P* Value
Mean ovulations	32.8 ± 4.8	33.6 ± 2.1	0.44
Mean ova/embryos	22.2 ± 5.2	17.0 ± 4.4	0.23
Ova + Embryos	133	136	
Total embryos	23 (17%)	73 (54%)	<0.0001
Mean embryos	3.8 ± 1.7	9.1 ± 3.1	
IETS grade1-2 embryos	16 (70%)	45 (62%)	0.62
Oocytes/ Uncleaved Zygotes	110 (83%)	63 (46%)	<0.0001

Conclusions

- FSH elevated during maternal aging as in women approaching menopause
- Higher proportions of ova/uncleaved zygotes in aged cows

Acknowledgements

- University of Saskatchewan for Scholarship
- SSR for Travel Grants
- NSERC and ADF Saskatchewan for Research Support

Effect of FSH Starvation on Competence of Bovine Oocytes *in vitro*

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WCVM

Introduction

- Previous studies from our laboratory showed that the bovine oocytes from early regressing follicles were more competent (P<0 05) than oocytes from growing or late regressing follicles. This is during the period of nadir in endogenous FSH (Sahmoune et al., 1999, Vasishta et al., 2003).
- Another study in cows demonstrated that 48-h of coasting produced significantly more competent oocytes than 24, 33 and 72-h of coasting (Blondin et al., 1997, 2002). However, this study used commercial FSH preparation containing LH activity. In addition, endogenous LH was also expected to be high. Therefore, it is not possible to differentiate between effect of FSH starvation and LH on oocyte competence.
- Coasting or FSH starvation is defined as the time interval between the cessation of exogenous FSH and the collection of oocytes.
- Coasting for 1-4 days did not affect the maturity, fertilization and embryo cleavage rate (Mikkelsen et al., 2003), but coasting beyond four days reduced implantation and pregnancy rate than shorter coasting intervals (Ulun Ulug et al., 2002).

Objectives

- To determine the effect of FSH starvation on competence of bovine oocytes *in vitro*.
- To determine the optimum time interval between FSH stimulation and oocyte collection to obtain maximum number of competent oocytes.

Hypothesis

- The time interval between FSH stimulation and oocyte collection influences oocyte competence.

Procedures

- Hereford-cross cows (n = 40) of 4-6 years of age were used.
- Ovulation was synchronized by administration of two $PGF_{2\alpha}$ injections 11 days apart.
- Follicular wave was synchronized by ablation of all follicles (≥5 mm) 5-7 days after ovulation.
- A functional corpus luteum was maintained throughout the period of treatment and oocyte collection.
- Transrectal ultrasonography was performed daily from ablation to oocyte collection.

Superstimulation and Oocyte Collection

- Heifers were given a total dose of 150 IU of purified bovine FSH (NIH S1 units) im, divided bid for 3 days (i.e. 6 treatments of 25 IU each).
- Cumulus-oocyte-complexes (COC) were collected by transvaginal ultrasound-guided follicle aspiration at 12, 48, 84 and 120 hours after the last FSH injection (n = 10/group).
- COC were matured and fertilized *in vitro*, and embryos were cultured *in vitro* for 7 days.

Statistical Analysis

- Blastocyst development was compared among groups by Fisher's exact test.

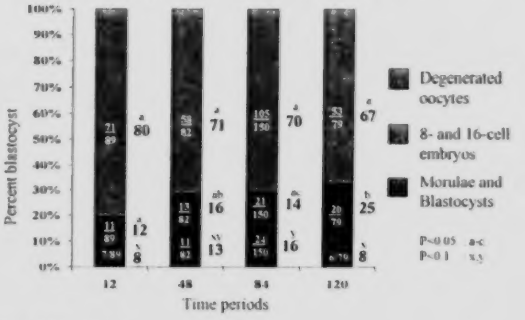
P<0 05 : Significant difference

P<0 1 : Tendency for a difference

Results

Time periods	n (cows)	# of follicles aspirated	# of COC collected
12	10	208	102
48	10	213	147
84	10	282	201
120	10	273	100
Total	**40**	**976**	**550**
Collection rate			56 %

Blastocyst Development Rate on Day 7



Conclusions

- A tendency towards higher blastocyst rate (P<0 1) at 48 and 84 hr than at 12 and 120 hr.
- More embryos (P<0 05) were arrested at 8- and 16-cell stage at 120 hr than 12 and 84 hr.
- FSH starvation *in vivo* seems to influence the developmental competence of bovine oocytes *in vitro*.

Supported by NSERC, SHRF and ADF Saskatchewan

Effect of FSH on Nuclear Maturation of Bovine Oocytes *in vivo*

D Saravanan, GP Adams, P Malhi, MM Mayes, MH Ratto and J Singh
Department of Veterinary Biomedical Sciences, Western College of Veterinary Medicine
University of Saskatchewan, 52 Campus Drive, Saskatoon SK Canada S7N 5B4

WCVM

Introduction

- High LH:FSH ratio affects ovarian response in cows (Gonzalez et al 1990; Schmidt et al 1991)
- Interval between end of superstimulatory treatment and oocyte collection appears to affect oocyte competence (Blondin et al 2002)
- LH (hCG) causes Ovarian Hyper-Stimulation Syndrome in some women (Devroey and Sirle 2004). It may be advantageous to treat such patients with purified FSH alone
- Purified bovine FSH is now available (Animal Endocrine Research Institute, LLC)

Objectives

- To determine if the time interval between end of FSH treatment and oocyte collection affects nuclear maturation of oocytes
- To determine if the duration of FSH treatment affects nuclear maturation of oocytes

Hypotheses

- FSH treatment (without LH) can initiate the nuclear maturation (Germinal Vesicle Break Down; GVBD) of oocytes
- Duration of FSH treatment will not affect the nuclear maturation status of oocytes
- FSH starvation in the absence of preovulatory LH surge will lead to completion of meiosis I (Oocytes in Metaphase II stage)

Procedures

- Hereford-cross cows (n = 20) 4-6 years of age were used
- Follicular wave synchronization was performed by ablation of all follicles (≥5 mm) 5-7 days after ovulation
- A functional corpus luteum was maintained throughout the period of treatment and oocyte collection
- Transrectal ultrasonography was performed daily from ablation to oocyte collection

Superstimulation and Oocyte Collection

- Treatment with purified bovine FSH (150 NIH S1 units total dose) was initiated 24 hours after ablation
- Cows were divided into two groups (n=10/group):
  - a. FSH every 12 hours for 3 days (6 doses, 25 S1 units/dose)
  - b. FSH every 12 hours for 4.5 days (9 doses, 16.7 S1 units/dose)
- Cumulus-Oocyte Complexes (COC) were collected by transvaginal ultrasound-guided follicle aspiration at 12, 48, 84, 120 hours after last FSH treatment in both groups (n=5 ovaries/time period)
- COC were classified under stereomicroscope
- Oocytes were fixed, stained with orcein stain and observed under microscope to determine nuclear status

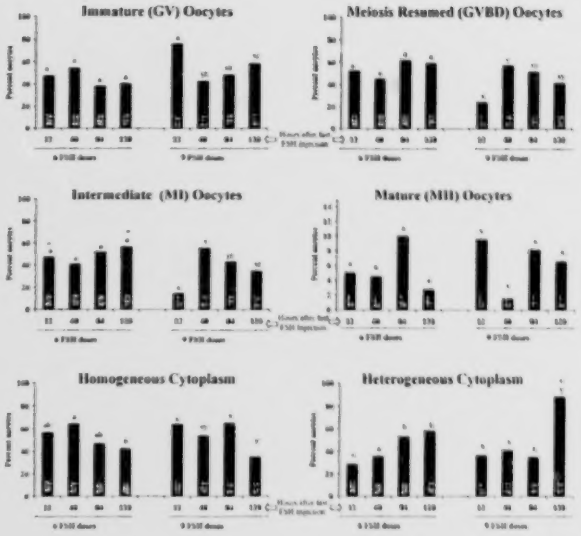
Statistical Analysis

- Nuclear status and appearance of cytoplasm of oocytes between 6 FSH and 9 FSH groups and among different time periods (12, 48, 84 and 120 hours) were statistically analyzed using Fisher's exact test
- $P<0.05$ was considered significant difference between groups

Results

Number of Oocytes Collected

FSH treatment	Oocyte collection Timing (hr)	No. of ovaries	No. of Follicles Aspirated	No. of COC collected	Collection rate
6 FSH doses	12	5	69	34	73%
	48	5	76	46	61%
	84	5	100	63	63%
	120	5	96	33	34%
9 FSH doses	12	5	69	36	53%
	48	5	134	71	44%
	84	5	93	48	51%
	120	5	133	61	46%
Total		40	771	420	54%



* Significant difference between treatment groups

Conclusions

- FSH treatment without LH can initiate nuclear maturation (GVBD) – Nearly 50% oocytes were observed to undergo GVBD
- Duration of FSH treatment did not affect the nuclear maturation status of oocytes
- FSH starvation (in the absence of preovulatory LH surge) did not lead to completion of meiosis I (Oocytes in Metaphase II stage) – Overall, only 6% of oocytes reached MII

Research supported by grants from the Saskatchewan Health Research Foundation, the Natural Sciences and Engineering Research Council of Canada and Agriculture Development Fund Saskatchewan

Others

(Publications and Abstracts)

Oocyte competence under different progestational environments

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Abstract

Prolonged treatment with progestagens for estrus synchronization results in poor fertility. The objectives of this study were to compare the developmental capacity of oocytes that develop 1) under low- versus high-progesterone concentrations, and 2) from persistent dominant follicles. Hereford crossbred heifers with a functional corpus luteum (CL) were assigned randomly to three groups (n=10/group) intended to provide low-, prolonged-low- and high- circulating progesterone (P4). Follicular wave emergence was synchronized among heifers by follicular ablation, and low P4 implants (once-used CIDR) were placed intravaginally. Heifers in low P4 and prolonged-low P4 groups were treated with 500μg PGF2α analog 12h apart on the day of ablation while heifers in high P4 group were permitted to retain their CL. Beginning 24h post-ablation, heifers in all groups were treated i.m. with 200mg FSH divided bid over 4 days (Day 0=first FSH treatment). Heifers in high P4 group were given PGF2α on Day 3. The CIDR devices were removed from heifers in low P4 and high P4 groups on Day 3, and from prolonged-low P4 group on Day 8. All heifers were treated i.m. with 25mg LH 24h after CIDR removal. Heifers were artificially inseminated at 24h and 36h post-LH treatment and were sent to slaughter 3 days after the last insemination. The embryos/ova were flushed from the uterine tubes. Ovarian ultrasonography was conducted on Days -1, 4, and 24h prior to slaughter. Data were analyzed using one-way ANOVA and Chi-squared tests. Plasma LH pulse frequency (36h before CIDR removal) was lowest and Day 3 P4 concentration was highest in high P4 group ($P<0.01$). The number of follicles ≥12mm was highest ($P=0.02$) and the number of follicles 9 to11 mm tended to be higher ($P=0.07$) in prolonged-low P4 group. No ovulations were detected in prolonged-low P4 group. The embryo/ovum recovery rate was higher ($P<0.01$) in high P4 (139/204, 68%) versus low P4 (109/218, 50%) group, but the proportion of fertilized ova was higher ($P<0.01$) in low P4 (94%) versus high P4 group (73%). It was concluded that in FSH treated heifers, oocytes that developed under a high-progesterone milieu were less capable of

developing into embryos, whereas oocytes that developed under a prolonged low- progesterone milieu failed to be ovulated.

1. Introduction

Early attempts at artificial insemination after estrus synchronization with progestagens (e.g., MGA in the feed or Synchro-Mate-B ear implants, Progesterone Releasing Intravaginal Device-PRID, etc.) resulted in poor fertility (Hansel et al., 1961; Hill et al., 1971; Henricks et al., 1973; Roche, 1974; Butcher and Pope, 1979; Beal et al., 1988; Brink and Kiracofe, 1988; Macmillan et al., 1991; Jochle, 1993; Savio et al., 1993a; Stock and Fortune, 1993; Wehrman et al., 1993). It has since been shown that after luteolysis, progestagen treatment results in a sub-luteal phase progestational influence, which i) allows a higher than normal pulse frequency of circulating luteinizing hormone (Ireland and Roche, 1982; Roberson et al., 1989) and consequent development of a persistent, over-sized dominant follicle (Stock AE and Fortune JE, 1993; Ahmad et al., 1995; Revah and Butler, 1996); and ii) blocks the positive feedback action of estradiol on hypothalamus for the induction of LH surge and consequent blockage of ovulation (Hobson and Hansel, 1972). On the contrary, the reduction in the pulse frequency of LH consequent to the luteal phase concentrations of progesterone (Rahe et al., 1980) results in the atresia of the dominant follicle (Lucy et al., 1992).

It has been documented that lower fertility following ovulation of an over-sized dominant follicle is not due to the uterine environment. The embryos recovered from normally developed follicles and transferred 7 days after estrus to the uterine horns of cows that ovulated persistent follicles resulted in pregnancy rates comparable to controls (Wehrman et al., 1997). Furthermore, the corpora lutea developed from persistent follicles produced progesterone comparable to controls (Stock and Fortune, 1993).

The persistence of an over-sized dominant follicle results in the prolonged period of estradiol secretion (Sirois and Fortune, 1990; Savio et al., 1993b). Prolonged exposure of a dominant follicle to estradiol was associated with the reduced fertility as a consequence of premature maturation of the oocyte (Mihm et al., 1994a; Revah and Butler, 1996; Austin et al., 1999). Hence, poor fertility following the ovulation of a persistent follicle may be due to the development of an oocyte under abnormal endocrine milieu consisting of high estrogenic environment (Sirois and Fortune, 1990; Savio et al., 1993b); higher LH pulse frequency (Roberson et al., 1989; Savio et al., 1993b) or formation of an abnormal embryo and early embryonic death (Mihm et al., 1994b; Ahmed et al., 1995; Revah and Butler, 1996).

In cattle, follicles in the terminal stages of development grow in a wave-like fashion that refers to a periodic and synchronous growth of a group of follicles. Cattle exhibit 2 or 3 waves of follicular activity during an interovulatory interval. During each wave, a single follicle is selected to outgrow the others in the cohort (called subordinate) and becomes a dominant follicle. Though, the dominant follicle of each wave has a capability to ovulate (Savio et al., 1990), only the dominant follicle of the last wave ovulates (Bergfelt et al., 1991; Lucy et al., 1992), i.e., a wave that produces dominant follicle during the progesterone decline consequent to the luteolysis is ovulatory. In the presence of the luteal phase progesterone, the dominant follicle undergoes atresia (Savio et al., 1993a).

The ovulatory follicle in 2-wave cycles develops under high-progesterone milieu for 7 days compared to 3 days in 3-wave cycles; and also the duration of its development is longer in 2- versus 3-wave cycles (11 versus 7 days, respectively; Ginther, et al., 1989). The shorter period of development of an ovulatory follicle in 3-wave cycles has been the basis of argument by many that the ovulatory follicle in 3-wave cycles are fertile compared to their 2-wave cycle counterpart (Ahmad et al., 1997; Townson et al., 2002; Bleach et al., 2004; Celik et al., 2005).

The relative competence of oocytes grown under rising or peak circulating progesterone (luteal phase follicular wave) and the one growing under declining concentrations of circulating progesterone (ovulatory follicular wave) is not known. Further, it is not clear whether poor fertility subsequent to progestagen treatment is a direct result of subnormal progesterone concentration and associated high LH pulse frequency and high estradiol concentration or secondarily, as a result of an ovulation of an aged oocyte. The present study was conducted with objectives 1) to compare the developmental capacity of oocytes grown under low versus high concentrations of progesterone, and 2) to determine the developmental competence of oocytes developed in the persistent, "over-dominant" follicles.

2. Materials and methods

All procedures described within were reviewed and approved by the University of Saskatchewan Protocol Review Committee, under the umbrella of the University Committee on Animal Care and Supply, and were performed in accordance with the principles outlined by the Canadian Council on Animal Care.

The experiment was carried out between June and August and heifers were maintained in outdoor corrals at the University of Saskatchewan's Goodale Research Farm (52°N and 106°W).

2.1. Animals

Forty nine Hereford crossbred heifers, 20 to 24 months of age and weighing 480 to 680 Kg were treated for ovulation induction using a single i.m. luteolytic dose of ProstaglandinF2α analog (PGF2α; 500 μg Cloprostenol; Estrumate, Schering-Plough Animal Health, Pointe-Claire, PQ, Canada). The study was designed such that all heifers had a functional CL at the beginning of the study (Fig.1). The first 30 heifers that ovulated, were assigned randomly to one of three

groups (n = 10/group) intended to provide low- (Group 1), prolonged-low- (Group 2), and high- (Group 3) plasma concentrations of progesterone (P4).

2.2. Experimental protocol

Follicular wave emergence was induced among heifers using ultrasound-guided ablation of follicles ≥5 mm between 5 to 8 days after ovulation. A silastic P4 implant "CIDR-B" (Controlled Intravaginal Drug Release device for Bovine; Vetrepharm Canada Inc., Belleville, ON, Canada) which had previously been used for about 9 days was placed in the vagina with the purpose of providing sub-luteal phase plasma concentrations of P4 (van Cleef et al., 1992). Heifers in the low- and prolonged-low P4 groups were treated with 2 luteolytic doses PGF2α 12 h apart and heifers in the high P4 group were permitted to retain their functional CL. Beginning 24 h post-ablation (i.e., at the expected time of new wave emergence, Bergfelt et al., 1994), heifers were treated with half the dose of the standard superstimulatory treatment (Looney et al., 1988; Wilson et al., 1989) that consisted of i.m. treatment with 200 mg Follicle Stimulating Hormone (FSH; Folltropin-V, Bioniche Animal Health Canada Inc., Belleville, ON, Canada) divided bid over 4 days. Heifers in the high P4 group were treated with 2 luteolytic doses of PGF2α 12 h apart on Day 3 (Day 0 = first FSH treatment). The CIDR devices were removed on Day 3 from heifers in the low- and high P4 groups, and on Day 8 from heifers in the prolonged-low P4 group. All heifers were treated with a single i.m. dose of Luteinizing hormone (LH; Lutropin-V, 25 mg, Bioniche Animal Health Canada Inc., Belleville, ON, Canada) 24 h after CIDR® removal. Heifers were inseminated artificially at 24 h and 36 h post-LH treatment and were sent to slaughter three days after the last breeding so as to collect the embryos from the uterine tubes (Guillomot, 1995).

2.3. Embryo collection

Embryo enters into the uterine horn from uterine tube around 4 to 5 days post-estrus (Guillomot, 1995). Hence, to facilitate ease in the collection of embryos from uterine tubes, heifers were sacrificed 3 days after estrus to recover embryos/ova from the uterine tubes.

Uterine tubes were dissected from the mesosalphinx to straighten them and were cut such that a small portion of the uterine horn was attached to them. By inserting an 18 gauge blunt needle, each uterine tube was flushed with 20 ml of phosphate buffered saline from the ampullary end toward the utero-tubal junction. Uterine horns were cut free from the broad-ligament and uterine end close to the bifurcation was blocked with a large tissue forceps. An incision was made to insert the tip of a 60 ml catheter-tip-syringe and horns were flushed individually from close to the bifurcation towards the utero-tubal junction with 75 ml of flushing media into the Emcon embryo filters. Ovaries were examined for corpora lutea to estimate the number of ovulations.

2.4. Ultrasonography

Ovarian ultrasound examinations were conducted using a 7.5-MHz linear-array transducer (Aloka SDD-900; Instruments for Science and Medicine, Vancouver, BC, Canada) on the day of ablation, 12 h after CIDR removal and 24 h prior to slaughter to monitor follicular development in response to different progestational treatments.

2.5. Blood sampling and radioimmunoassay

Jugular blood samples were collected in heparinized tubes (10 ml; Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ) on the day of ablation, on the last of FSH injection and, just before LH injection to monitor LH and P4 concentrations. Starting at 36 h before CIDR removal, serial blood sampling was conducted (n=4/group) at 15 min interval for 8 h using jugular vein catheterization to determine the LH pulse frequency and amplitude. Blood was

centrifuged for 15 min at 1500 x g within 30-60 min of collection. Plasma was aspirated and stored at -20°C until assayed using radio immunoassay for P4 and luteinizing hormone.

Plasma concentrations of LH were measured using a double antibody radioimmunoassay and were expressed as NIDDK-bLH4 units (Evans et al., 1994; Honaramooz et al., 1998). The range of the standard curve was 0.06 to 8 ng/ml. The intra-assay coefficient of variation was 6.8% and 1.9% for the low (mean, 0.43 ng/ml) and high (mean, 0.99 ng/ml) reference samples, respectively. The LH-pulse frequency, pulse amplitude, means, and basal concentrations were calculated (Honnramooz et al., 1998). Plasma P4 concentrations were determined (Kastelic et al., 1999) using a solid-phase radioimmunoassay (catalogue # TKPG5; Lot # TKPG21443; Coat-A-Count; Diagnostic Products Corporation, Los Angeles, CA). The range of the standard curve was 0.1 to 40 ng/ml). The intrassay coefficients of variation were 6.2% (low reference), 7.9% (medium reference), and 3.1% (high reference).

2.6. Data analyses

Comparison of the ratio of unfertilized-ova:embryos, and different stages of embryos, between groups 1 and 3 will address the objective 1 and the one between groups 1 and 2 will address the objective 2. Recovery rates of ova/embryos, ratio of ova to embryos, proportion of different stage embryos were compared between groups 1 and 3 using a 2 x 2 chi-squared contingency table. Effects of group (groups 1 to 3) and day for the hormonal levels and number of different size follicles were analyzed by analysis of variance for repeated measures using the mixed procedure (Littell et al., 2000). Five covariance structures (compound symmetry; autoregressive, order 1; unstructured; unstructured 1; and Huynh-Feldt) were fitted to the data and the best model was selected based on the smallest Akaike information criterion values. If main effects or their interaction were statistically significant ($P \leq 0.05$), multiple comparisons were made using Tukey post hoc test. Correlation between the LH and P4 profiles was estimated

using Pearson correlation analysis. All analyses were performed using Statistical Analysis System software package (SAS learning edition 2.0; SAS Institute Inc., Cary, NC).

3. Results

3.1. Hormonal profile

Heifers of respective groups exhibited distinct endocrinological characteristics in accordance with the induced progestational environments. The characteristics of LH pulses on Day 2 in low and high P4 groups, and Day 7 in prolonged-low P4 group were compared to confirm the efficacy of differential P4 treatments (Table 1). Similarly, blood plasma samples were collected on Day 3 and also 24 h before breeding to monitor circulating concentrations of LH and P4.

The LH pulse amplitude (mean ± SE) did not differ among groups ($P = 0.23$). However, the LH pulse frequency per 8 h was lowest in high P4 group compared to low and prolonged-low P4 groups (Low P4 group: 6.00±0.00; Prolonged-low P4 group: 5.25±0.85; and High P4 group: 1.75±0.48; $P < 0.01$). The plasma LH concentration (mean ± SE) on Day 3 did not differ among groups ($P = 0.46$; Fig.2). Though, statistically non-significant ($P = 0.39$), the plasma LH concentrations 12 h before breeding were low in prolonged-low P4 group versus low P4 and high P4 groups. The circulating LH concentrations elevated between Day 3 and 12 h before breeding within low P4 ($P = 0.09$), prolonged low P4 ($P = 0.01$), and high P4 ($P = 0.13$) groups. In accordance with the induced P4 milieu, the plasma P4 concentrations (mean ± SE) on Day 3 and 12 h before breeding were higher in high P4 versus low and prolonged-low P4 groups ($P < 0.01$; Fig.3). Within groups, there was a significant decline ($P < 0.01$) in the circulating P4 concentrations in response to removal of CIDR devices. Though, there was a strong inverse relationship between LH and P4 profiles in all treatment groups (Low P4 group: $r = -0.91$;

Prolonged-low P4 group: r = -0.99; and High P4 group: r = -0.97), the relationship was statistically non-significant (P > 0.10).

Table 1

Comparison of characteristics of LH pulses among groups

Hormonal profile (mean±SE)	Low P4	Prolonged-low P4	High P4
Basal concentration	0.0182 ± 0.01^{a}	0.004 ± 0.002^{ab}	0.000 ± 0.00^{b}
Pulse amplitude	0.20 ± 0.05^{a}	0.17 ± 0.02^{a}	0.34 ± 0.11^{a}
Pulse frequency	6.00 ± 0.00^{a}	5.25 ± 0.85^{a}	1.75 ± 0.48^{b}

[ab] values in a row with no common superscripts are different (P < 0.05)

3.2. Follicular profile

Comparison of follicle numbers in different size categories 24 h prior to breeding (equivalent to Day 4 in low and high P4 groups and Day 9 in prolonged-low P4 group; Fig.4) revealed highest number of follicles ≥12 mm (P = 0.02) in prolonged-low P4 group. The prolonged-low P4 group also tended (P = 0.07) to have the highest number of follicles 9 to 11 mm in diameter. However, there was no difference (P > 0.39) in the total number of follicles and follicles <9 mm in diameter among groups.

In the prolonged-low P4 group, the number of follicles within follicle size category did not differ (P > 0.14; Fig.5) between Days 4 and 9. However, a reduction in follicle numbers in the larger size category was followed by an increase of equal magnitude in the preceding lower size category of follicles. For example, with a reduction in the number of follicles ≥12 mm and

6-8 mm, there was a concomitant increase of equal magnitude in the number of follicles ≥9 mm and ≤5 mm, respectively.

3.3. Ova/embryo recovery

No ovulations were detected and no oocytes were recovered from heifers in the prolonged-low P4 group, hence, this group's data were excluded from analyses related to ova/embryo recovery.

There was no difference (P = 0.83) among low P4 and high P4 groups for the mean total number of corpora lutea i.e., ovulations (Low P4 group: 21.80±4.37; and High P4 group: 20.40±4.72; Fig. 6) and for the proportion of different stage embryos with respect to the total embryos recovered (P > 0.10; Fig. 6). The ovum/embryo recovery rate was higher (P<0.01) in high P4 group (68%) versus low P4 group (50%), but the proportion of fertilized ova was higher (P<0.01) in the latter (94% versus 73%; Table 2). The proportion of ova/embryos recovered from the uterine tube was lower in low P4 versus high P4 group (P < 0.01; Table 2).

Table 2

Comparison of response to superstimulatory treatment as well as rate of ovum/embryo transport in the genital tract under low versus high P4 environments

	Low P4	High P4
Ovum/embryo recovery rate	109/218 (50%)[a]	139/204 (68%)[b]
Proportion of fertilized ova	102/109 (94%)[a]	102/139 (73%)[b]
Proportion of ova/embryos recovered from uterine tube	17/111 (15%)[a]	51/139 (37%)[b]

[ab]values in a row with no common superscripts are different (P < 0.05)

4. Discussion

Present study compared the *in vivo* developmental competence of oocytes being developed under the low- versus high P4 environments and determined the developmental competence of oocytes that were subjected to extended period of development by providing sub-luteal phase plasma P4 concentrations. Results revealed that oocytes that developed under a high P4 milieu were less capable of developing into embryos, whereas oocytes that were subjected to extended period of development failed to be ovulated. The developmental competence of oocytes did not differ under low- and high P4 environments. The study also revealed that ova/embryos that were developed under low P4 milieu are transported to the uterine horns much faster than the ones developed under high P4 milieu.

In the low- and prolonged-low P4 groups, the plasma P4 concentrations were low and LH pulse frequency was high, whereas in the high P4 group, the plasma P4 was high and the LH pulse frequency was low. The treatment protocol was effective in inducing the intended hormonal milieu which matched with that of previous studies wherein circulating P4 concentrations were manipulated (Ireland and Roche, 1982; Roberson et al., 1989; Sirois and Fortune, 1990; Savio et al., 1993b; Stock and Fortune, 1993; Custer et al., 1994; Kinder et al., 1996; Binelli et al., 1999; Vinoles et al., 1999).

The follicular profile and ovulation rate did not differ between the low- and high P4 groups. This indicates that the response to FSH treatment under low and high progestational environment is comparable, which is in agreement with an earlier study wherein the superstimulatory treatment at the beginning of wave 1 (low P4) or wave 2 (high P4) resulted in a similar response in terms of number of large follicles and ovulations (Adams et a., 1994). In another study (Desaulniers et al., 1995) wherein the cows (low P4) and heifers (high P4) were superstimulated, the follicular development in response to the superstimulatory treatment was

comparable. It was stated that P4 environment affects LH pulse frequency which in turn influences estradiol production and not the follicular development.

The low ovum/embryo recovery rate (number of ova/embryos recovered out of total number of ovulations detected) found in low P4 group versus high P4 group is in agreement with a recent study (Silva et al., 2002) that compared ovum/embryo production in response to superstimulatory treatment in cows (low P4) and heifers (high P4). However, the low ovum/embryo recovery in the low P4 group in the present study contrasted earlier study, which did not find any difference (Adams et al., 1994). Such discrepancy in the low P4 group may be due to loss of ova/embryos consequent to the faster rate of transport of ova/embryos from the uterine tubes to the uterine horns.

The higher recovery rate of fertilized ova in the low P4 group versus high P4 group is in contrast to the findings of earlier studies (Adams et al., 1994; Silva et al., 2002). However, the higher fertilization rate in the low P4 group may be attributed to the higher pulsatile secretion of LH that regulates the availability of aromatizable substrate for the production of estradiol by granulosa cells of developing follicles (Lucy et al., 1992). The increased secretion of estradiol by a dominant follicle during the growing stage is indicative of its functional development (Badinga et al., 1992; Price et al., 1995; Stewart et al., 1996), whereas the reduced secretion of estradiol is indicative of atresia (Lucy et al., 1992; Savio et al., 1993). Additionally, the high estradiol and low P4 environment during proestrus and estrus maintains the specific pattern of protein synthesis and secretions by oviductal regions that are conducive to the fertilization process (Binelli et al., 1999). Specific protein synthesis in the oviduct is required for efficient egg transport within the uterine tube (Fuentealba et al., 1988), oviductal epithelial cell proliferation (Slayden et al., 1994), sperm capacitation (Anderson and Killian, 1994), and sperm/egg binding (Staros and Killian, 1998). The receptor protein for LH has been identified in the uterine tube of cattle and an in-vitro study has demonstrated the dose-dependent secretion of oviductal proteins

in response to the treatment of bovine oviductal epithelial cells with hCG (Sun et al., 1997). Hence, there is a possibility that high frequency of LH pulses in the low P4 group may have created oviductal environment suitable for the normal fertilization.

The recovery of similar proportion of different stage embryos from group of heifers that were exposed to low- versus high P4 environments confirm that the differential P4 milieu before ovulation affects the fertilizing capacity of oocytes but not the developmental competence of fertilized ova. Normal P4 secretion is required for sustaining pregnancy and improving the pregnancy rate (Mann et al., 1999; Silva, 2002) and the P4 secretion from the corpora lutea formed from follicles developed under different endocrine milieu was comparable (Stock and Fortune, 1993).

Altering the interval from follicular wave emergence to progestin removal does not affect the synchrony of estrus or ovulation (Utt et al., 2003). Hence, the complete anovulation in all 10 heifers that were exposed to prolonged sub-luteal phase P4 may be due to the functional loss of dominance and induction of atresia in large follicles. In earlier studies in cattle (Sirois and Fortune, 1990; Adams et al., 1992) and ewes (Vinoles et al., 1999), the functional dominance of a persistent follicle was evident from the low number of total follicles compared to controls. The functional loss of dominance of follicles in prolonged-low P4 group heifers can be explained from the decrease in the number of follicles in larger size categories with concomitant increase in the preceding size category of follicles (Fig.6), when number of follicles in different size categories were compared at two intervals. The possibility of the luteinization of follicles may be ruled out because had there been a luteinization, there would have been an increase in the circulating progesterone concentrations.

Selected growing follicles manifest LH receptor protein on granulosa cells (Bao et al., 1997; Stewart et al., 1996; Beg et al., 2001) and becomes very sensitive to alterations in LH (Beg et al., 2001). In response to higher LH concentrations, the selected follicles actively secret

estradiol (Badinga et al., 1992; Price et al., 1995; Stewart et al., 1996), however, the estradiol secretion is reduced in the atretic follicles (Lucy et al., 1992; Savio et al., 1993). Exposure to P4 increases intra-follicular content of lower molecular mass insulin-like growth factor binding proteins (IGFBPs) that enhances apoptosis of granulosa cells in dominant follicles (Manikkam and Rajamahendran, 1997). In the present study, the heifers were superstimulated with FSH to obtain a large number of oocytes as a study material to determine the effect of prolonged sub-luteal phase P4 environment. However, ovarian response to superstimulation is associated with reduction in the proportion of follicles that are ovulated (Saumande and Chupin, 1986) consequent to the reduction in the responsiveness of follicles to LH (Saumande and Chupin, 1986). The estradiol secretion in the FSH-stimulated prolonged-multiple follicles decline to trace amounts (Revah and Butler, 1996), which may be due to the competition among follicles for available LH (Adams et al., 1993). In the present study, heifers were treated with normal dose of LH for ovulation induction, however, the dose of LH required to ovulate smaller follicles is higher than required for the larger follicles (Sartori et al., 2001).

5. Conclusions

The exposure of oocytes to short-term high-progesterone environment *in vivo* affects their fertilizing capacity. However, the endocrine environment prevailing before fertilization does not affect the developmental competence of fertilized ova. Multiple follicles developed under prolonged-low progesterone environment fail to ovulate.

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References:

Adams, G.P., Kot, K., Smith, C.A. and Ginther, O.J., 1993. Selection of a dominant follicle and suppression of follicular growth in heifers. Anim Reprod Sci, 30: 259-271.

Adams, G.P., Matteri, R.L. and Ginther, O.J., 1992. Effect of progesterone on oarian follicles, emergence of follicular waves and circulating follicle-stimuating hormone in heifers. J Reprod Fertil, 95: 627-640.

Adams, G.P., Nasser, L.F., Bo, G.A., Garcia, A., Del-Campo, M.R. and Mapletoft, R.J., 1994. Superovulatory response of ovarian follicles of wave 1 versus wave 2 in heifers. Theriogenology, 42: 1103-13.

Ahmad, N., Schrick, F.N., Butcher, R.L. and Inskeep E.K., 1995. Effects of persistent follicles on early embryonic losses in beef cows. Biol Reprod, 52: 1129-1135.

Ahmad, N., Townsend, E.C., Dailey, R.A. and Inskeep, E.K., 1997. Relationship of hormonal patterns and fertility to occurrence of two or three waves of ovarian follicles, before and after breeding, in beef cows and heifers. Anim Reprod Sci, 49: 13-28.

Anderson, S.H. and Killian, G.J., 1994. Effect of macromolecules from oviductal conditioned medium on bovine sperm motion and capacitation. Biol Reprod, 51: 795-799.

Austin, E.J., Mihm, M., Ryan, M.P., Williams, D.H. and Roche, J.F., 1999. Effect of duration of dominance of the ovulatory follicle on onset of estrous and fertility in heifers. J Anim Sci, 77: 2219-2226.

Beal, W.E., Chenault, J.R., Day, M.L. and Corah, L.R., 1988. Variation in conception rates following synchronization of estrus with melengesterol acetate and prostaglandin F2α. J Anim Sci, 66: 599-602.

Bergfelt, D.R., Kastelic, J.P. and Ginther, O.J., 1991. Continued periodic emergence of follicular waves in nonbred progesterone-treated heifers. Anim Reprod Sci, 24: 193-204.

Bergfelt, D.R., Lightfoot, K.C. and Adams, G.P., 1994. Ovarian synchronization following ultrasound-guided transvaginal follicle ablation in heifers. Theriogenology, 42: 895-907.

Binelli, M., Hampton, J., Buhi, W.C. and Thatcher, W.W., 1999. Persistent dominant follicle alters pattern of oviductal secretory proteins from cows at estrus. Biol Reprod, 61: 127-134.

Bleach, E.C.L., Glencross, R.G. and Knight, P.G., 2004. Association between ovarian follicle development and pregnancy rates in dairy cows undergoing spontaneous oestrous cycles. Reprod, 127: 621-629.

Brink, J.T. and Kiracofe, G.H., 1988. Effect of estrus cycle stage at Synchro-Mate-B treatment on conception and time to estrus in cattle. Theriogenology, 29: 513-518.

Butcher, R.L. and Pope, R.S. 1979. Role of estrogen during prolonged estrous cycles of the rat on subsequent embryonic death or development. Biol Reprod, 21: 491-495.

Celik, H.A., Aydin, I., Sendag, S. and Dinc, D.A., 2005. Number of Follicular waves and their effect on pregnancy rate in the cow. Reprod Dom Anim, 40: 87-92.

Desaulniers, D.M., Lussier, J.G., Goff, A.K., Bousquet, D., Guilbault, L.A., 1995. Follicular development and reproductive endocrinology during and after superovulation in heifers and mature cows displaying contrasting superovulatory responses. Domest Anim Endocrinol, 12: 117-131.

Evans, A.C.O., Adams, G.P. and Rawlings, N.C., 1994. Endocrine and ovarian follicular changes leading up to the first ovulation in prepubertal heifers. J Reprod Fertil, 100: 187-194.

Fuentealba, B., Nieto, M. and Croxatto, H.B., 1988. Estrogen and progesterone receptors in the oviduct during egg transport in cyclic and pregnant rats. Biol Reprod, 39: 751-757.

Ginther, O.J., Knopf, L. and Kastelic, J.P., 1989. Temporal associations among ovarian events in cattle during oestrous cycle with two or three follicular waves. J Reprod Fertil, 87: 223-230.

Guillomot, M., 1995. Cellular interactions during implantation in domestic ruminants. J. Reprod. Fertil. Suppl. 49, 39-51.

Hansel, W., Malven, P.V. and Black, D.L., 1961. Estrous cycle regulation in the bovine. J Anim Sci, 20:621-625.

Henricks, D.M., Hill, J.R. and Dickey, J.F., 1973. Plasma ovarian hormone levels and fertility in beef heifers treated with melegesterol acetate (MGA). J Anim Sci, 37: 1169-1175.

Utt, M.D., Jousan, F.D. and Beal, W.E., 2003. The effects of varying the interval from follicular wave emergence to progestin withdrawal on follicular dynamics and the synchrony of estrus in beef cattle. J Anim Sci, 81: 1562-1567.

Sun, T., Lei, Z.M. and Rao, C.V., 1997. A novel regulation of the oviductal glycol-protein gene expression by luteinizing hormone in bovine tubal epithelial cells. Mol Cell Endocrinol, 131: 97-108.

Townson, D.H., Tsang, P.C.W., Butler, W.R., Frajblat, M., Griel, L.C., Johnson Jr, C.J., Milvae, R.A., Niksic, G.M. and Pate, J.L., 2002. Relationship of fertility to ovarian follicular waves before breeding in dairy cows. J. Anim Sci, 80: 1053-1058.

van Cleef, J., Lucy, M.C., Wilcox, C.J. and Thatcher, W.W., 1992. Plasma and milk progesterone and plasma LH in ovariectomized lactating cows treated with new or used controlled internal drug release devices. Anim Reprod Sci, 27: 91-106.

Vinoles, C., Meikle, A., Forsberg, M. and Rubianes, E., 1999. The effect of subluteal levels of exogenous progesterone on follicular dynamics ad endocrine pattern during the early luteal phase of the ewe. Theriogenology, 51: 1351-1361.

Wehrman, M.E. Pike, K.E., Melvin, E.J., Kojima, F.N. and Kinder J.E., 1997. Development of a persistent ovarian follicle and associated elevated concentrations of 17β-estradiol preceding ovulaytion does not alter the pregnancy rate after embryo transfer in cattle. Theriogenology, 47:1413-1421.

Hill, J.R., Lamond, D.R., Henricks, D.M., Dickey, J.F. and Niswender, G.D., 1971. The effect of melengestrol acetate (MGA) on ovarian function and fertilization in beef heifers. Biol Reprod, 4: 16-22.

Hobson, W.C. and Hansel, W., 1972. Plasma LH levels after ovariectomy, corpus luteum removal and estradiol administration in cattle. Endo, 91: 185-190.

Honaramooz, A., Chandolia, R.K., Beard, A.P. and Rawlings, N.C., 1998. Excitatory amino acid regulation of gonadotropin secretion in prepubertal heifer calves. Biol Reprod, 59: 1124-1130.

Ireland, J.J. and Roche, J.F., 1982. Effect of progesterone on basal LH and episodic LH and FSH secretion in heifers. J Reprod Fertil., 64: 295-302.

Jochle, W., 1993. Forty years of control of the oestrous cycle in ruminants: progress made, unresolved problems and the potential impact on sperm encapsulation technology. Reprod Fertil and Develop, 5: 587-594.

Kastelic, J.P., Olson, W.O., Martinez, M., Cook, R.B. and Mapletoft, R.J., 1999. Synchronization of estrus in beef cattle with norgestomet and estradiol valerate. Can Vet J, 40: 173-178.

Littell, R.C., Pendergast, J. and Natarajan, R., 2000. Modelling covariance structure in the analysis of repeated measures data. Stat Med, 19: 1793-1819.

Lucy, M.C., Savio, J.D., Badinga, L., De-La-Sota, R.L. and Thatcher, W.W., 1992. Factors that affect ovarian follicular dynamics in cattle. J Anim Sci, 70: 3615-3626.

Looney, C.R., Bondioli, K.R., Hill, K.G. and Massey, J.M., 1988. Superovulation of donor cows with bovine follicle-stimulating hormone (bFSH) produced by recombinant DNA technology. Theriogenology, 29:271 (abstract).

Macmillan, K.L., Taufa, V.K., Barnes, D.R. and Day, A.M., 1991. Plasma progesterone concentrations in heifers and cows treated with a new intravaginal device. Anim Reprod, 26: 25-40.

Manikkam, M. ad Rajamahendran, R., 1997. Progesterone-induced atresia of the proestrous dominant follicle in the bovine ovary: changes in diameter, insulin-like growth factor system, aromatase activity, steroid hormones, and apoptotic index. Biol Reprod, 57: 580-587.

Mann, G.E., Starbuck, G.R. and Lamming, G.E., 1999. Progesterone treatment inhibit the secretion of oestradiol by the first follicle wave in the cow. J Reprod Fertil Abstract Ser 24, Abstr# 31, pp 16.

Mihm, M., Baguisi, A., Boland, M.P. and Roche, J.F., 1994a. Association between the duration of dominance of the ovulatory follicle and pregnancy rate in beef heifers. J Reprod Fertil, 102: 123-130.

Mihm, M., Currant, N., Hytell, P., Boland, M.P. ad Roche, J.F.,1994b. Resumption of meiosis in cattle oocytes from pre-ovulatory follicles with a short and long duration of dominance. J Reprod Fertil Abstr ser, 13-14.

Rahe, C.H., Owens, R.E., Fleeger, J.L., Newton, H.J. and Harms, P.G., 1980. Pattern of plasma luteinizing hormone in the cyclic cow: dependence upon the period of the cycle. Endo, 107: 498-503.

Revah, I. and Butler, W.R., 1996. Prolonged dominance of follicles and reduced viability of bovine oocytes. J Reprod Fertil, 106: 39-47.

Roberson, M.S., Wolfe, M.W., Stumpf, T.T. Kittock, R.J. and Kinder J.E., 1989. Luteinizing hormone secretion and corpus luteum function in cows receiving two levels of progesterone. Biol Reprod., 41: 997-1003.

Roche, J.F., 1974. Synchronization of oestrus in heifers with implants of progesterone. J Reprod Fertil, 41:337-344.

Savio, J.D., Boland, M.P., Hynes, N. and Roche, J.F., 1990. Resumption of follicular activity in the early postpartum period of dairy cows. J Reprod Fertil, 88: 569-579.

Savio, J.D., Thatcher, W.W., Morris, G.R., Entwistle, K., Drost, M. and Mattiacci, M.R., 1993a. Effects of induction of low plasma progesterone concentrations with a progesterone-releasing intravaginal device on follicular turnover and fertility. J Reprod Fertil, 98: 77-84.

Savio, J.D., Thatcher, W.W., Badinga, L., de la Sota, R.L. and Wolfenson, D., 1993b. Regulation of dominant follicle turnover during the oestrous cycle in cows. J Reprod Fertil, 97: 197-203.

Silva, J.C.e., Costa, L.L.da. and Silva, J.R., 2002. Embryo yield and plasma progesterone profiles in superovulated dairy cows and heifers. Anim Reprod Sci, 69: 1-8.

Sirois, J. and Fortune, J.E., 1988. Ovarian follicular dynamics during the estrous cycle in heifers monitored by real-time ultrasonography. Biol Reprod, 39: 308-317.

Sirois, J. and Fortune, J.E., 1990. Lengthening the bovine estrous cycle with low levels of exogenous progesterone: A model for studying ovarian follicular dominance. Endo, 127: 916-925.

Slayden, O.D. and Brenner, R.M., 1994. RU 486 action after estrogen priming in the endometrium and oviducts of rhesus monkeys (*Macaca mulatta*). J Clin Endocrinol Metab, 78:440-448.

Staros A.L. and Killian, G.J., 1998. In vitro association of six oviductal fluid proteins with the bovine zona pellucida. J Reprod Fertil, 112: 131-137.

Stock, A.E. and Fortune J.E., 1993. Ovarian follicular dominance in cattle: relationship between prolonged growth of the ovulatory follicle and endocrine parameters. Endo, 132: 1108-1114.

Wehrman, M.E. Roberson, M.S., Cupp, A.S., Kojima, F.N., Stumpf, T.T., Werth, L.A., Wolfe, M.W., Kittok R.J. and Kinder J.E., 1993. Increasing exogenous progesterone during synchronization of estrus decreases endogenous 17β-estradiol and ncreases conception in cows. Biol Reprod, 49: 214-220.

Wilson, J.M., Moore, K., Jones, A.L. and Looney, C.R., 1989. Recombinant bovine follicle stimulating hormone: Dose and duration regimens for superovulation of embryo donors. Theriogenology, 31: 273 (abstract).

Captions for figures:

Fig. 1. Experimental protocol. Details in the materials and methods, section 2.2.

Fig. 2. Circulating plasma concentrations of LH (mean ± SE) on Day 3 (Day 0 = 1st FSH treatment) and 24 h before Artificial insemination in heifers treated to induce low, prolonged-low and high progesterone environments. ^{ab}within a day, means (±SEM) with different superscripts differed ($P < 0.05$); ^{xy}within a treatment, means (±SEM) with different superscripts differed ($P < 0.05$).

Fig. 3. Circulating plasma concentrations of progesterone (mean ± SE) on Day 3 (Day 0 = 1st FSH treatment) and 24 h before Artificial insemination in heifers treated to induce low, prolonged-low and high progesterone environments. ^{ab}within a day, means (±SEM) with different superscripts differed ($P < 0.05$); ^{xy}within a treatment, means (±SEM) with different superscripts differed ($P < 0.05$).

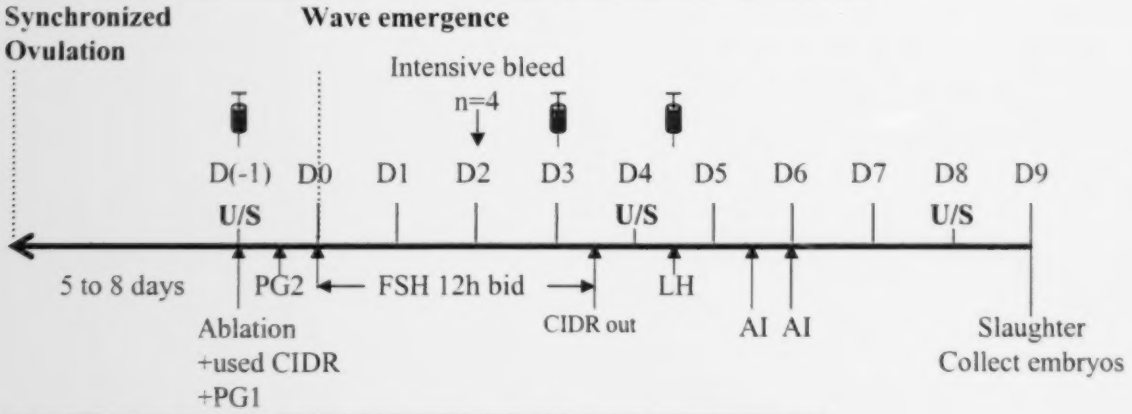
Fig.4. Number of follicles in different size categories among low, prolonged-low and high progesterone groups 12 h before treating heifers with LH for ovulation induction. [a,b] within follicle categories, means (±SEM) with different superscripts differed ($P < 0.05$).

Fig.5. Comparison of follicle numbers in different size categories in prolonged-low progesterone group on Day 4 (Day 0 = first FSH treatment) versus Day 9 i.e., 12 h before treating heifers with LH for ovulation induction. [a,b]within follicle categories, means (±SEM) with different superscripts differed ($P < 0.05$).

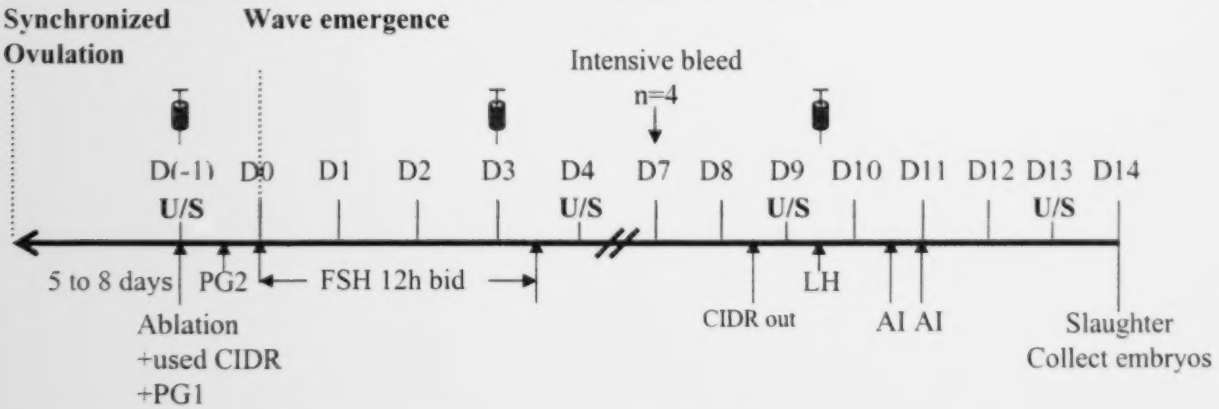
Fig. 6. Number of corpora luteal (CL, representing ovulations), ova/embryos recovered, and embryos of different developmental stages (mean ± SE) recovered from heifers that were superstimulated under the low- (n= 10) and high- (n = 10) progesterone milieu.

[a,b]within categories, means (±SEM) with different superscripts differed ($P < 0.05$).

Group 1 (n = 10): Low P4 (simulated to preovulatory dominant follicle)



Group 2 (n = 10): Low P4 (simulated to persistent dominant follicle)



Group 3 (n = 10): High P4 (simulated to luteal phase dominant follicle)

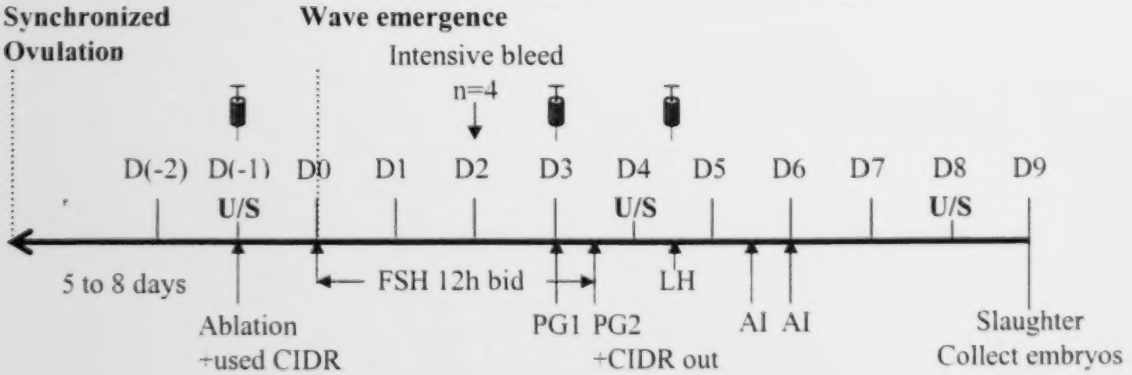
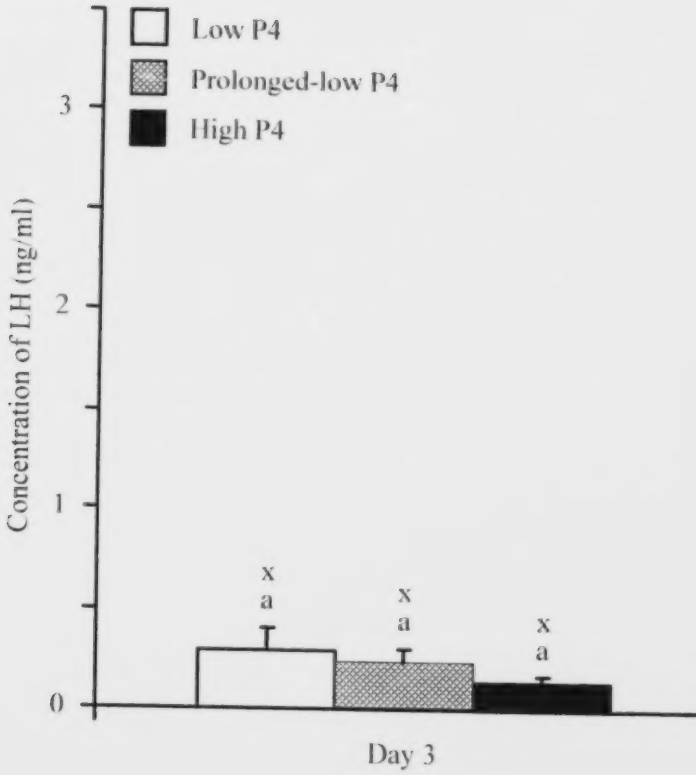
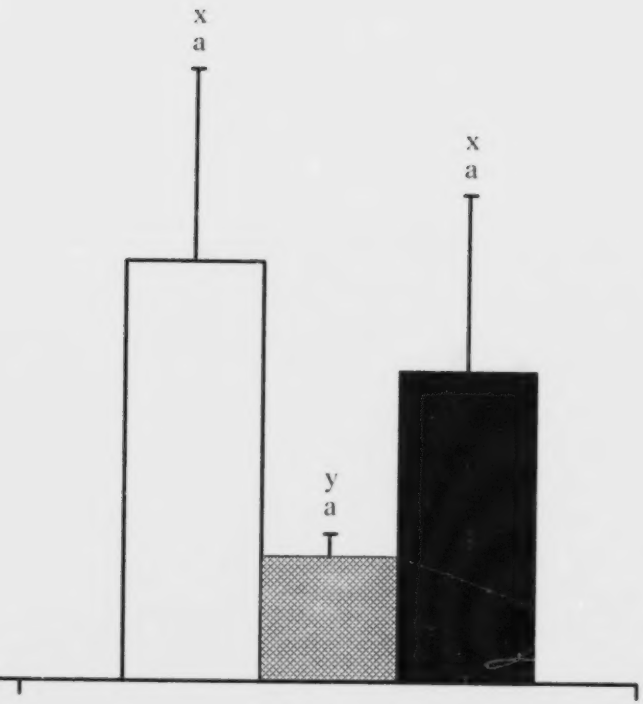


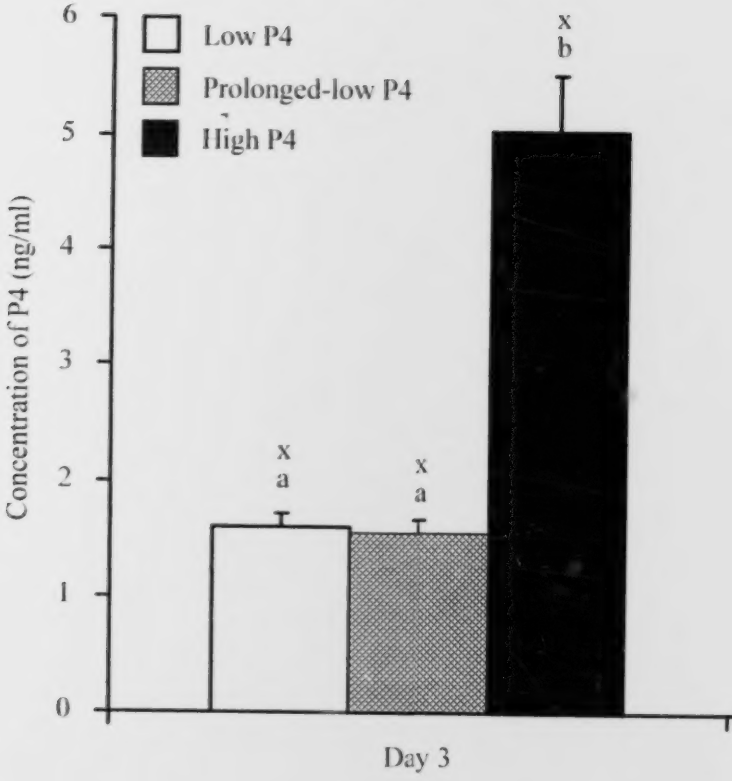
Fig.2

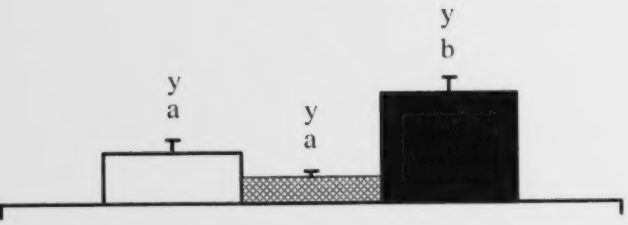

Low P4
Prolonged-low P4
High P4
Concentration of LH (ng/ml)
3
2
1
0
x
a
x
a
x
a
Day 3



24 h before AI

Fig.3


Low P4
Prolonged-low P4
High P4
Concentration of P4 (ng/ml)
0
1
2
3
4
5
6
x
a
x
a
x
b
Day 3



24 h before AI

Fig.4

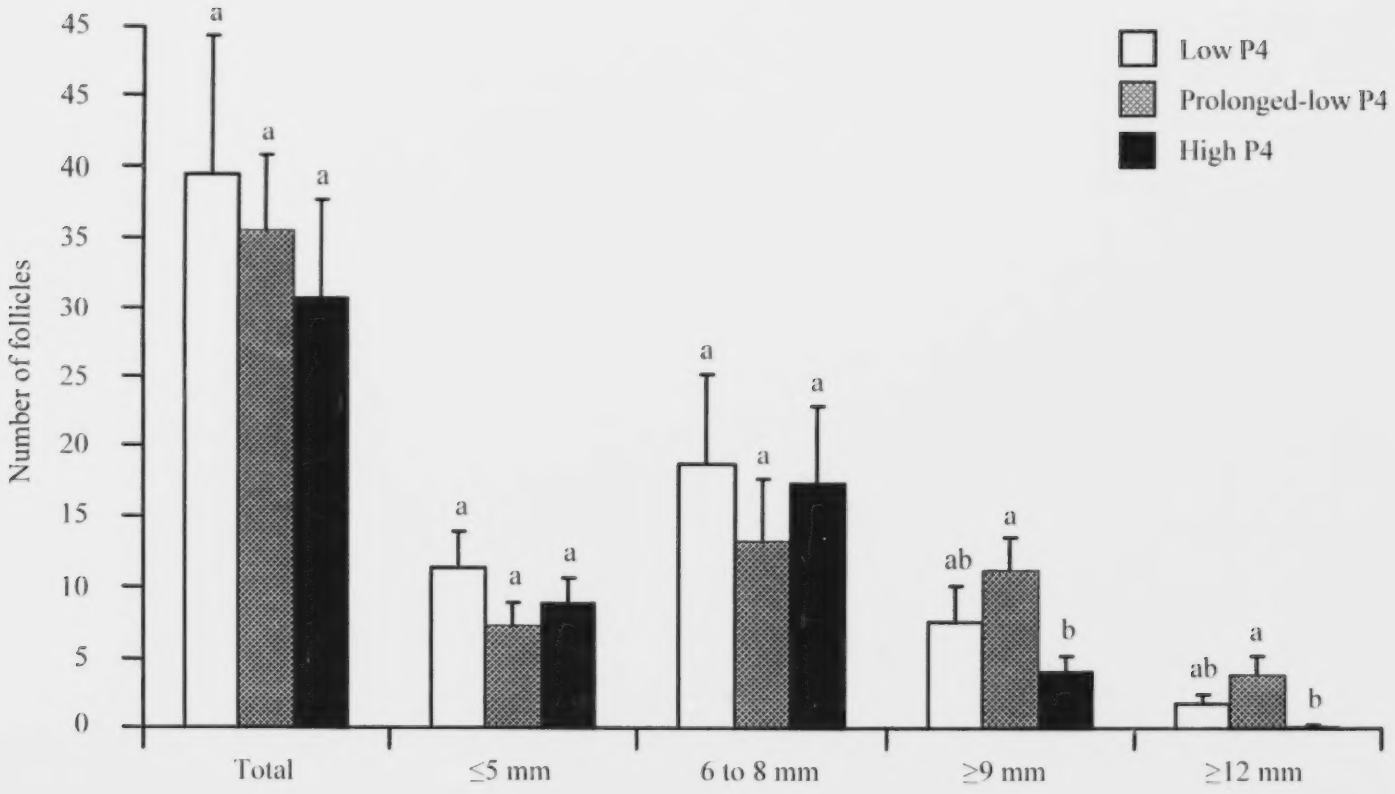

Low P4
Prolonged-low P4
High P4
Number of follicles
45
40
35
30
25
20
15
10
5
0
a
a
a
a
a
a
a
a
a
ab
a
b
ab
a
b
Total
≤5 mm
6 to 8 mm
≥9 mm
≥12 mm

Fig.5

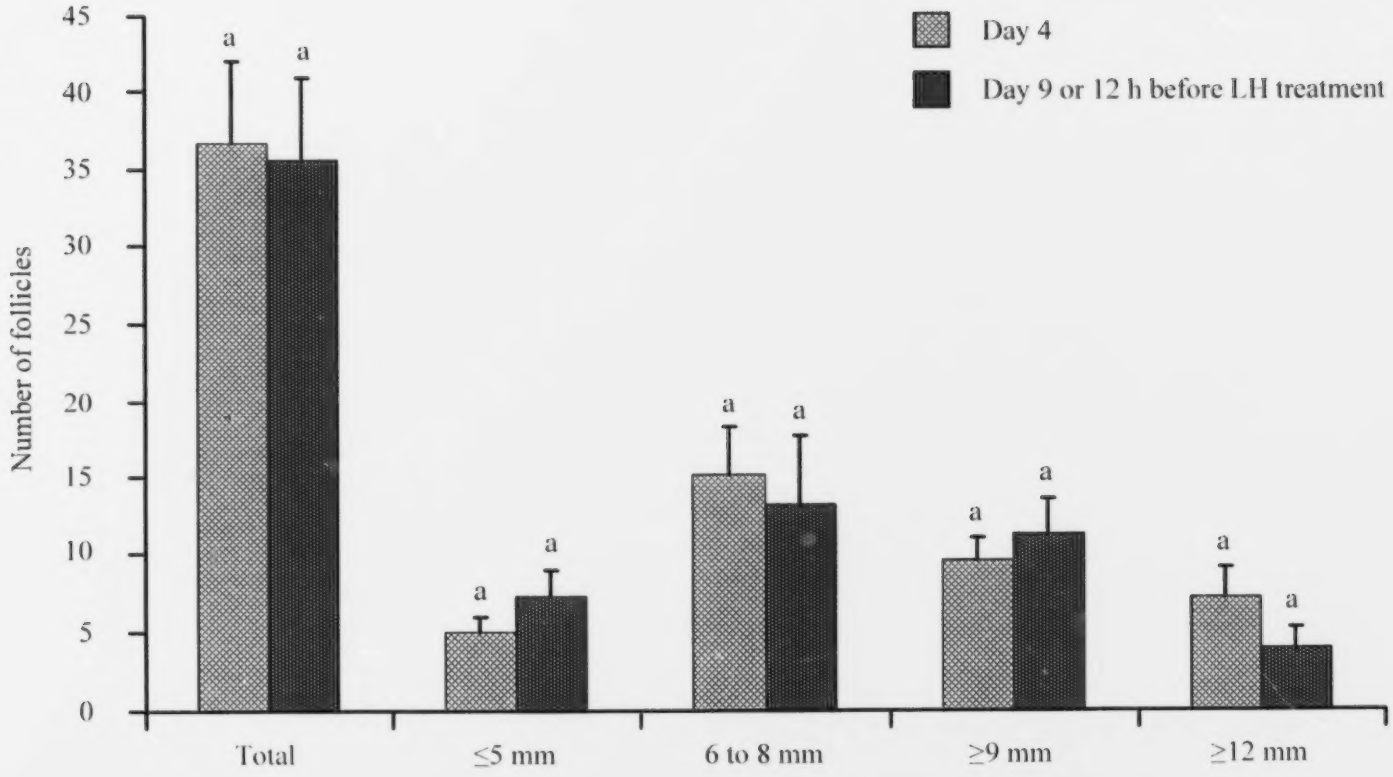
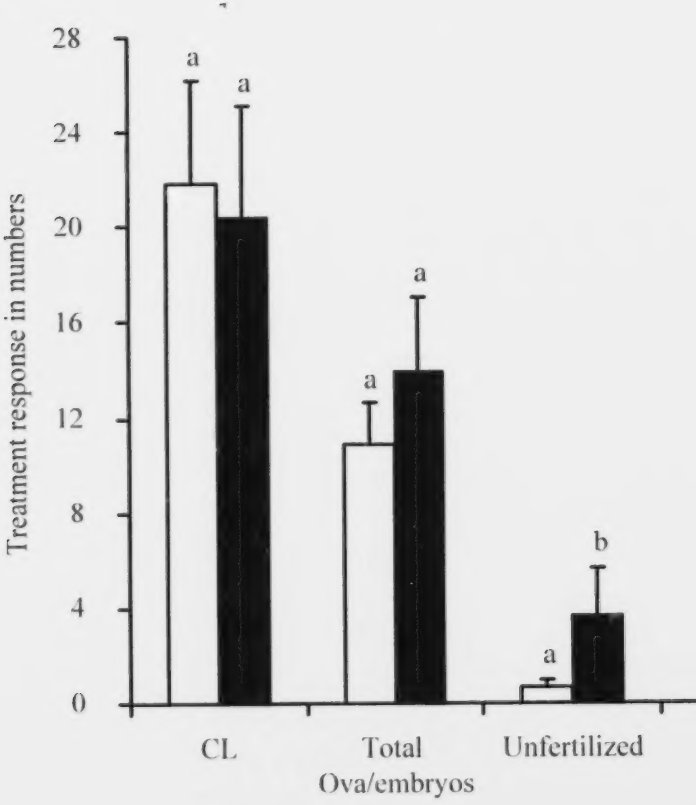
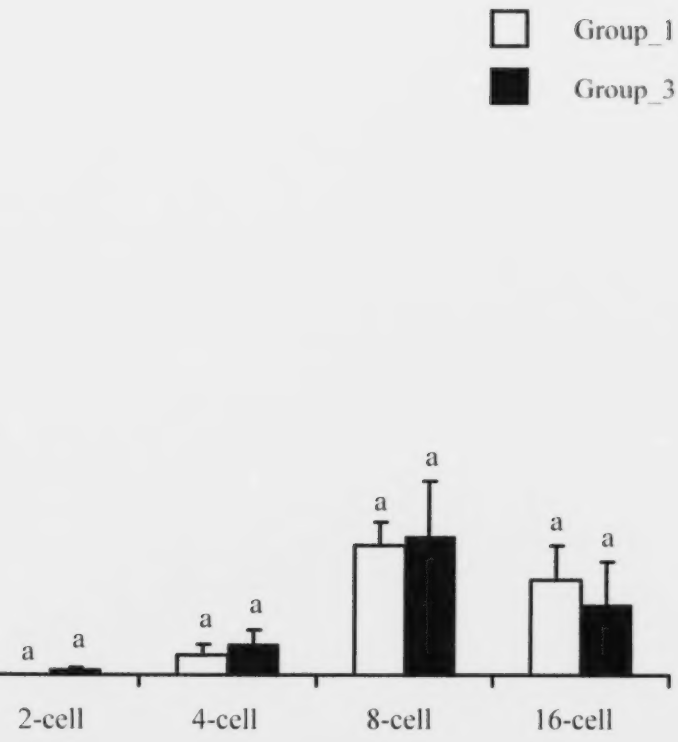

Day 4
Day 9 or 12 h before LH treatment
Number of follicles
45
40
35
30
25
20
15
10
5
0
a
a
a
a
a
a
a
a
a
a
Total
≤5 mm
6 to 8 mm
≥9 mm
≥12 mm

Fig.6


28
24
20
16
12
8
4
0
Treatment response in numbers
a
a
a
a
a
b
CL
Total
Unfertilized
Ova/embryos


Group_1
Group_3
a
a
a
a
a
a
a
a
2-cell
4-cell
8-cell
16-cell

Proceedings of the 2nd Annual Reproductive Science and Medicine Research Symposium
University of Saskatchewan Saskatoon February 24, 2006

OOCYTE COMPETENCE UNDER DIFFERENT PROGESTATIONAL ENVIRONMENTS

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Objectives: To compare the developmental capacity of oocytes that develop 1) under low- versus high-progesterone concentrations, and 2) from persistent dominant follicles.
Study Methods: Hereford crossbred heifers (n=30) with a functional corpus luteum were assigned randomly to three groups (n=10/group) intended to provide low (group-1), prolonged-low (group-2) and high (group-3) circulating progesterone. Follicular wave emergence was synchronized among heifers by follicular ablation, and once-used progesterone implants (CIDR-B) were placed intravaginally. Heifers in groups-1 and -2 were treated with Prostaglandin-F2α 12h apart on the day of CIDR placement. Beginning 24h post-ablation, heifers in all groups were treated i.m. with 200mg FSH divided bid over 4 days (Day 0=first FSH treatment). On Day 3, heifers in group-3 were treated with Prostaglandin-F2α 12h apart. The CIDR devices were removed from heifers in groups-1 and -3 on Day 3, and from group-2 on Day 8. All heifers were treated i.m. with 25mg LH 24h after CIDR removal. Heifers were artificially inseminated at 24h and 36h post-LH treatment and were sent to slaughter 3 days after the last insemination. The embryos/ova were flushed from the uterine tubes. Ovarian ultrasound examinations were conducted on Days -1, 4, and 24h prior to slaughter. Blood samples were collected on Days -1, 3, and just before LH treatment. Starting at 36h before CIDR removal, serial blood samples were collected (n=4/group) at 15-minute intervals for 8h. Data were analyzed using one-way ANOVA and Chi square tests.
Results: Plasma LH pulse frequency was lowest and progesterone concentration was highest in group-3 ($P<0.01$). Prior to ovulation, the number of follicles ≥12mm was highest ($P=0.02$) in group-2. Group-2 also tended ($P=0.07$) to have the highest number of follicles 9-11mm in diameter. The embryo/ovum recovery rate was higher ($P<0.01$) in group-3 (68%) versus group-1 (50%), but the proportion of fertilized ova was higher ($P<0.01$) in the latter (94% versus 73%). No ovulations were detected from heifers in group-2.
Conclusions: In superstimulated heifers, oocytes that developed under a high-progesterone milieu were less capable of developing into embryos, whereas oocytes that developed under a prolonged low- progesterone milieu failed to be ovulated.

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Reproduction Fertility and Development 18: 290

The bovine model for reproductive aging: superovulatory response

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Two experiments were done to test the hypotheses that aging in cattle is associated with a reduction in the number of follicles recruited into an ovarian follicular wave, and a reduction in the ovarian response following gonadotropin treatment for superovulation. Old cows (13 to 16 years old; n = 6 to 9) and their young daughters (3 to 6 years old; n = 7 to 9) were treated to induce an ovarian superstimulatory response twice in each of 2 consecutive years (i.e., 31 and 33 superstimulatory cycles, respectively). In Experiment 1, follicular wave emergence was synchronized using a single treatment of estradiol (5 mg) combined with progesterone (100mg) at the time of CIDR-B (Bioniche Animal Health, Canada) insertion. Superstimulatory treatment consisted of a total dose of 50 mg NIH-FSH-P1 (Folltropin-V; Bioniche Animal Health, Canada) per 100 kg body weight divided into twice daily intramuscular injections over 4 days. CIDR were removed at the time of last FSH treatment and 500 μg cloprostenol (Estrumate; Schering-Plough Animal Health, Canada) was administered twice along with last 2 FSH treatments to induce luteolysis. Ovulation was induced using 25 mg porcine LH (Lutropin-V; Bioniche Animal Health, Canada) administered 24 hr after the last FSH treatment. Ovulations were detected at 24, 48 and 72 hr after LH treatment using ultrasonography. In Experiment 2, follicular wave emergence was synchronized with ultrasound-guided follicle ablation in the presence of a functional corpus luteum. Superstimulatory treatment was given as in Experiment 1. Ultrasound-guided follicle aspirations were performed 48 to 72 hr after the last FSH for the purposes of another experiment. In both experiments, the ovarian follicular response was monitored daily by ultrasonography. There was no difference ($P > 0.3$) in mean body weight and dose of FSH treatment between old cows and their daughters. Fewer 2-5 mm follicles ($P < 0.01$) were detected at the expected time of wave emergence in old cows than in their daughters. In Experiment 1, the difference in mean number of ovulations in old and young cows (32 ± 4 versus 40 ± 3) did not reach significance ($P = 0.11$), but fewer follicles ≥ 6 mm ($P < 0.01$) were detected on the day before ovulation in old cows than in their daughters. The number of follicles ≥ 6 mm detected just before ovulation (Experiment 1) or follicular aspiration (Experiment 2) in successive superstimulatory cycles was correlated within animals ($r > 0.8$; $P < 0.0001$). Similarly, the number of ovulations in successive superstimulatory cycles (Experiment 1) was correlated within animals ($r = 0.6$; $P = 0.04$). In conclusion, aging was associated with fewer 2-5 mm follicles at the time of wave emergence, a lower follicular response and a tendency for a lower ovulatory response subsequent to gonadotropin treatment. The follicular and ovulatory response to superstimulatory treatment was repeatable within individuals.

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Theriogenology 64:778

Bovine Model to Study Age-Associated Infertility in Women: Developmental Competence of Oocytes

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At present, there is no well characterized animal model to study reproductive aging in women. This experiment was designed to study the effect of age on oocyte developmental competence in cattle, and to validate a bovine model for the study of reproductive aging in women. We hypothesize that maternal aging in cattle is associated with reduced oocyte developmental competence. In year 1, 13-15 year-old cows (n=6) and their 3-5 year-old young daughters (n=8) were superstimulated with exogenous FSH and LH. The cows in both age groups were artificially inseminated. Embryos were collected non-surgically, evaluated and transferred in pairs into synchronized 2-5 year old recipient cows (n=32). The experiment was repeated in year 2 with same group of old cows (n=9; 14-16 years old) and their daughters (n=9; 3-6 years old), but the embryos were transferred both as singletons and pairs (67 recipient cows; age, 2-5 year). Ultrasonically detected ovulations, recovery of ova/embryos, and embryo survival were compared by Student's t–test and Fisher's exact test. The mean number of ovulations per donor (31±4 versus 38±3; P=0.15) and mean ova/embryos recovered (21±4 versus 20±3; P=0.8) did not differ between old cows and their daughters, respectively. However, fewer embryos (P=0.04) were recovered from old cows (6±2) than their daughters (12±2). Similarly, the proportion of oocytes/uncleaved zygotes collected from old cows was higher (P<0.0001) than their daughters (222/312, 71% versus 119/316, 38% respectively). The proportion of IETS grade 1-2 embryos (59/90, 66% versus 133/197, 68%; P=0.9) did not differ between age groups. Survival of embryos in recipients on Day 45 of gestation did not differ between old and young cows (24/57, 42% versus 46/96, 48%; P=0.5). The reduced recovery of embryos and higher proportion of oocytes/uncleaved zygotes in old cows than their daughters supports the hypothesis that aging is associated with reduced developmental competence of oocytes.

Key words: Aging, Bovine model, Oocyte

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Proceedings of the annual meeting of the Society for the Study of Reproduction, Vancouver, BC August 2004 Abstract 35

EFFECT OF MATERNAL AGE ON GONADOTROPIN SECRETION AND OOCYTE COMPETENCE IN A BOVINE MODEL

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Maternal aging is associated with hormonal changes and reduced fertility in women. At present, there is no good animal model to study maternal aging in women. The results of recent studies document the utility of the bovine model for the study of follicular dynamics in women. The objective of this study was to validate a bovine model for study of the effects of aging in women. We hypothesized that maternal aging is associated with altered gonadotropin secretion and reduced oocyte competence in cattle. In Experiment 1, the ovaries of old cows (age, 13-15 years; n=10) and their daughters (age, 1-4 years; n=9) were examined daily for one estrous cycle using ultrasonography to record follicular dynamics. Plasma samples were obtained every 12 hr for the determination of FSH and LH concentrations. In Experiment 2, old cows (n=6) and daughters (n=8) were superstimulated with Folltropin-V and artificially inseminated. Embryos were collected non-surgically, evaluated and transferred in pairs into synchronous recipients (n=32; age, 2-5years). Detected ovulations and recovery rates of ova/embryos were recorded. Data were compared by ANOVA for repeated measures and Fisher's exact test. Mean plasma FSH concentrations during the estrous cycle were higher ($P<0.05$) in old cows than in their daughters, while LH concentrations did not differ ($P=0.4$). The total number of ovulations per donor (32.8±4.8 vs. 33.6±2.1; $P=0.44$) and ova/embryos recovered (22.2±5.2 vs. 17.0±4.4; $P=0.23$) did not differ among old cows and their daughters. However, the proportion of IETS grade 1-2 embryos (16/133 vs. 45/136; $P<0.0001$) and grade 3-4 embryos (7/133 vs. 28/136; $P=0.0002$) recovered from old cows were lower than their daughters. The proportion of oocytes/uncleaved zygotes collected from old cows was higher than that of their daughters (110/133, 83% vs. 63/136, 46%; $P<0.0001$). Embryo survival rates did not differ between old and young cows (7/14 vs. 20/46; $P=0.76$). In conclusions, endocrine changes observed in old cows are similar to that reported for women approaching menopause. A significantly higher proportion of oocytes/uncleaved zygotes from old cows are suggestive of a defect in fertilization or processes immediately after fertilization.

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University of Saskatchewan Saskatoon, February 24, 2006

OOCYTE MEIOTIC MATURATION IN A BOVINE MODEL OF REPRODUCTIVE AGING

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Objectives

To test the hypothesis that meiotic maturation (ability to complete first meiotic division) is compromised in oocytes of old cows. The role of LH in meiotic maturation was also studied.

Study methods

In Experiment 1, superstimulatory treatment with FSH was initiated in mother-daughter pairs (14-15yr and 3-5yr respectively, n=7 pairs) after follicle wave synchronization. A functional CL was maintained throughout the experiment. Follicles were aspirated from one ovary 5 days after wave emergence. LH was given immediately thereafter. Follicles were aspirated from the other ovary 22hr later. Oocytes meiotic stage was evaluated after aceto-orcein staining. In Experiment 2, 14-17yr old cows (n=10) and their 3-7yr old daughters (n=12) were superstimulated as described previously. Luteolysis was induced with PGF2α 3.5 days after wave emergence followed by LH 32hr later. Follicles were aspirated 18-20hr after LH treatment. Oocytes from cows with majority of expanded and compact COC were matured in-vitro for 6hr and 24hr respectively. Meiotic stage was evaluated after Giemsa staining of chromosomal spreads. The data were compared by Student t-test and Chi-Square test.

Results

Numbers (mean±SEM) of ≥6mm follicles on the day of oocyte retrieval did not differ (P=0.12) among old and young cows in Experiment 1 (27±6 versus 38±3) but differed (P=0.01) in Experiment 2 (30±3 versus 47±5). Concentrations of LH and progesterone in both experiments did not differ (P>0.4) between age groups. In Experiment 1, most of the oocytes recovered from old and young cows before (48/52 versus 71/75; P=0.6) or after (58/61 versus 61/66; P=0.5) LH exposure were in germinal vesicle or germinal vesicle breakdown stage. In Experiment 2, old cows had higher (P=0.02) numbers of expanded COC (68/182 versus 95/350) at collection, and a tendency for higher (P=0.08) numbers of metaphase II (69/115 versus 101/203) after 6hrs of in-vitro incubation. Luteolysis and LH treatment (Experiment 2) resulted in higher numbers of metaphase II (170/318 versus 2/66; P<0.01) than LH treatment during high progesterone (Experiment 1).

Conclusions

Age did not effect oocyte meiotic maturation in this group of highly fertile old cows. Luteolysis before LH exposure is an important factor for in-vivo oocyte meiotic maturation.

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EFFECT OF FSH STARVATION ON THE DEVELOPMENTAL COMPETENCE OF BOVINE OOCYTES IN VITRO.

Saravanan D, Mayes M.A., Adams G.P., Singh J.
Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon S7N 5B4, Canada.

The objectives of this study were to determine the effect of in vivo FSH starvation on the competence of bovine oocytes to develop in vitro, and to determine the optimum time interval between FSH stimulation and oocyte collection. A follicular wave was induced during the mid-luteal phase in Hereford-cross heifers (n=40) by ablation of all follicles ≥ 5 mm in diameter. Heifers were given a total dose of 150 IU of purified bovine FSH (NIH S1 units) im, divided bid for 3 days (i.e. 6 treatments of 25 IU each). Cumulus-oocyte-complexes (COC) were collected by transvaginal ultrasound-guided follicle aspiration at 12, 48, 84 and 120 hours after the last FSH injection (n = 10/group). The COC were matured and fertilized in vitro, and embryos were cultured in vitro for 7 days. Blastocyst development was compared among groups by Fisher Exact test. A total of 976 follicles were aspirated and 550 COC were obtained from 40 heifers. The proportion of oocytes that developed into blastocysts tended to be greater ($P<0.1$) in the 48 hr (13%) and 84 hr groups (16%) than in the 12 hr (8%) and 120 hr groups (8%). A greater proportion ($P<0.05$) of 8- and 16-cell embryos was obtained from the 120 hr group (25%) than in the 12 hr (12%) and 84 hr groups (14%). In conclusion, the study supports the hypothesis that FSH starvation influences oocyte competence and optimum time for oocyte collection appears to be 48 and 84 hours after last FSH injection.

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The objectives of this study were to determine the effect of FSH starvation on the developmental competence of bovine oocytes in vitro, and to determine the optimum time interval between FSH stimulation and oocyte collection. A follicular wave was induced during mid-luteal phase in Hereford-cross heifers (n=40) by ablation of all follicles > 5 mm in diameter. All heifers received 6 injections of purified bovine FSH at 12 hr interval (total dose = 150 NIH S1 units). Cumulus-oocyte-complexes (COC) were collected by transvaginal ultrasound aspiration at 12, 48, 84 and 120 hours after last FSH injection (n = 10/group). COCs were graded, matured in vitro and subjected to in vitro fertilization. The percentage of blastocyst was determined after 7 days of in vitro embryo culture. Percent blastocyst development was analyzed by Fisher Exact test and $P \leq 0.05$ was considered significant difference. A total of 976 follicles were aspirated and 550 COC were obtained from 40 heifers. Proportion of oocytes that developed into blastocysts tended to be greater ($P \leq 0.1$) in 48 (13.4%) and 84 hours (16%) than in 12 (7.9%) and 120 hour (7.6%) groups. Greater proportion ($P \leq 0.05$) of 8- and 16-cell stage embryos was obtained at 120 hours (25.3%) than at 12 (12.3%) and 84 hours (14%). In conclusion, the study supports the hypothesis that FSH starvation influences oocyte competence and optimum time for oocyte collection appears to be 48 and 84 hours after last FSH injection.

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Effect of FSH on nuclear maturation of oocytes in the bovine model

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Objectives: The objectives of this study were to determine the effects of duration of FSH treatment for superstimulation and time interval thereafter on nuclear maturation of oocytes from large (≥9 mm) and small (≤8 mm) follicles.

Methods: Emergence of follicular wave was synchronized in cows (4-6 years) by ablation of ≥ 5 mm follicles at day 5-7 after ovulation (Day 0). A functional corpus luteum was maintained throughout the experiment. All animals received equal total dose of purified bovine FSH; group 1 (n=10/per group) received 6 doses (25 NIH-S1 units every 12 hours) and group 2 received 9 doses (16.7 NIH S1 units every 12 hours) starting at the expected time of wave emergence. Cumulus oocyte complexes (COC) were collected by transvaginal ultrasound-guided needle aspiration at 12, 48, 84, and 120 hours after last FSH injection. COCs were classified and oocytes were fixed and stained with orcein stain. Nuclear status of oocytes was classified as immature (germinal vesicle stage), intermediate (germinal vesicle breakdown to metaphase I) and mature (metaphase II). Data were analyzed using Fisher's exact test.

Results: Overall, 51.2% (173/338) oocytes displayed GVBD or more advanced stage of nuclear maturation. There were more immature oocytes at 12 hours than 48 hours in 9 doses group (76% vs. 42.9%), respectively; $P< 0.05$), however there was no difference ($P>0.05$) between 12, 84 and 120 hours. There were more intermediate oocytes at 12 and 120 hours in the 6 doses group than the 9 doses group (47.5% vs. 14.3% and 56.8 vs. 34.8%, respectively; $P<0.05$). More ($P<0.05$) oocytes with heterogeneous cytoplasm were recorded at 120 hours in 9 doses group than all other time periods.

Conclusions: The study concluded that FSH without LH could initiate break down of germinal vesicle. Duration of FSH treatment did not affect the nuclear status of oocytes. However, FSH starvation (in the absence of preovulatory LH surge) did not lead to completion of meiosis I (oocyte in Metaphase II stage).

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Effect of FSH on nuclear maturation of bovine oocytes in vivo

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The study was conducted to determine if the duration of FSH treatment and FSH starvation affect the nuclear maturation of bovine oocytes. Emergence of follicle wave was synchronized (n=20 cows) by ablation of ≥ 5mm follicles 5-7 days after ovulation. The design allowed to maintain a functional corpus luteum throughout the period of FSH treatment and oocyte collection. One group of cows (n=10 per group) received 6 doses of FSH (25 S1 units every 12 hours) and the other group received 9 doses (16.7 S1 units every 12 hours). Cumulus-Oocyte Complexes (COC) were collected by transvaginal ultrasound needle puncture technique at 12, 48, 84 and 120 hours after last FSH injection. COCs were classified and nuclear status of oocyte was recorded after orcein staining. Nuclear status and appearance of cytoplasm were statistically analyzed using Fisher's exact test. Nearly 50% of oocytes underwent germinal vesicle breakdown and no major difference was observed between the two treatment groups. The study concluded that FSH treatment without LH can initiate nuclear maturation (GVBD), however, the treatment did not lead to completion of meiosis I. Duration of FSH treatment did not affect nuclear maturation status of oocytes.

KEYWORDS: Ovarian follicles, oocyte maturation, cumulus-oocyte complex, cattle, gonadotropin treatment

Ovarian follicle development during the seasonal transition in wapiti

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Wapiti are seasonally polyestrus. The transition into and out of the breeding season is marked by resumption of ovulation in the autumn and cessation of ovulation in the winter. The objective of this study was to characterize follicular and luteal dynamics in wapiti during the transitional periods. The transition from anestrus to estrus was monitored in 15 hinds, aged 2 to 14 years, over two successive seasons (11 in first season and 9 in second; n=20 observations) on a farm near Saskatoon (52 07'N, 106 38'W). The transition from estrus to anestrus was monitored in 11 hinds over one season (n=11 observations). The ovaries were examined daily using transrectral ultrasonography and a B-mode ultrasound machine with a 7.5 MHz linear array transducer. In 17 of 20 observations, the duration of the first interovulatory interval (IOI, 9.1±.3 days) was less than half the reported 21-day cycle. With one exception, short IOI were characterized by 1 wave of follicular development. The duration of the ovulatory season was 138±9.7 days (range, 74 to 182 days). The median date of the last ovulation of the breeding season was

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A simple ultrasound test to predict the superstimulatory response in cattle

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Abstract

We tested the hypotheses that: (1) the superstimulatory response is related to the intrinsic number of follicles recruited into a follicular wave; and (2) the number of follicles recruited into a wave is correlated to the number of follicles recruited into the successive wave. A positive correlation will form the basis of a test for predicting the superstimulatory response. Cows ($n = 141$) were treated with estradiol and progesterone to synchronize follicular wave emergence (first synchronization) and ranked according to the number of follicles ≥ 2 mm at wave emergence to select the upper and lower 10% of the herd. Follicular wave emergence was synchronized again in the high-end ($n = 16$) and low-end ($n = 20$) groups (second synchronization), and cows were treated with FSH twice daily for 3 days. High-end cows had a greater number of follicles ($P < 0.001$) than low-end cows at the time of wave emergence after both the first and second synchronizations in the 2–3 and 4–6 mm categories. The numbers of 2–3 and 4–6 mm follicles at wave emergence after the first and second synchronizations were positively correlated ($P < 0.001$; $r = 0.77$ and 0.71, respectively). Endogenous FSH peak at the time of wave emergence was higher in the low-end group than in the high-end group. Superstimulatory treatment resulted in more than double the number of follicles ($P < 0.003$) in the 5–7 mm and ≥ 8 mm categories in the high-end group than in the low-end group (16.8 ± 2.2 versus 8.1 ± 0.9 and 22.7 ± 4.1 versus 9.7 ± 1.6, respectively). The number of follicles ≥ 5 and ≥ 8 mm at the end of superstimulation was positively correlated ($P < 0.001$) with the total number of follicles ≥ 2 mm at the time of wave emergence after both the first ($r = 0.64$ and 0.54, respectively) and second ($r = 0.65$ and 0.5, respectively) synchronizations. Based on the results of this study, the superstimulatory response can be predicted by the number of follicles ≥ 2 mm at wave emergence. For practical purposes, practitioners can expect the number of follicles ≥ 5 mm after ovarian superstimulation to be approximately 71% of the number of follicles ≥ 2 mm at the time of wave

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emergence. Results validated the proposed simple ultrasound-based test for predicting the superstimulatory response of individual cows.
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Keywords: Cattle; Follicular dynamics; Superstimulation; Superovulation; Ultrasonography; Wave emergence

1. Introduction

Ovarian superstimulation is induced primarily in donor cows for embryo transfer or for oocyte collection and in vitro embryo production. The goal of superstimulatory treatment is to induce the growth of multiple follicles to produce multiple competent oocytes capable of developing into transferable embryos. In a study of over 2000 beef cows [1], an average of six transferable embryos were produced per treatment; however, 30% of the cows yielded 70% of embryos, while 24% of the cows produced no embryo. Similar variability was reported for a group of 987 dairy cows [2]. The authors of another large, epidemiological study [3] concluded that the superovulatory response is not a heritable trait, that the future response cannot be predicted by previous response, and that "environmental" factors play a large role in the variability in the superovulatory response. Extreme variability in the superstimulatory response remains an enigma and a major limitation in the profitable and efficient implementation of embryo technology in cattle [4,5].

Efforts to optimize the superstimulatory response in cattle have involved removal of excess LH from pituitary preparations of FSH, the use of recombinant bovine FSH, neutralizing antibodies against eCG, and numerous adjustments in the dose, route, and frequency of gonadotropin treatments [6–16]. In spite of these efforts, extreme inconsistency in response persists and some have postulated that a major source of variability results from differing status of the follicular wave at initiation of treatment [17,18]. The superstimulatory response is inferior after selection of the dominant follicle [19–26]. A greater superovulatory response was obtained when superstimulatory treatment was initiated at the time of follicular wave emergence rather than 1 or 2 days later [27], and the superovulatory response of Wave 1 (rising progesterone) was similar to that of Wave 2 (high-endogenous progesterone) [28]. Ablation of the dominant follicle before superovulatory treatment improved the number of viable embryos per session from 3.9 to 5.4 in cows but not in heifers (reviewed in [19]). As a result of these findings, superstimulatory protocols now incorporate synchronization of the follicular wave, either by estradiol-progesterone treatment or follicular ablation, before initiation of ovarian superstimulatory treatment [17,27,29–32].

Transient surges in circulating concentrations of FSH were found to be responsible for eliciting periodic emergence of follicular waves [33–35]. Results of one study led authors to hypothesize that superstimulatory gonadotropin treatment rescues follicles of an existing wave from atresia rather than recruit new follicles into the wave [34]. In this regard, initial findings from separate and independent studies suggest a link between the variability in the number of follicles present at the time of wave emergence and the responsiveness to superstimulatory treatment [31,36,37]. In addition, follicle recruitment after repeated follicle puncture had high repeatability [38].

In this study, two hypotheses were tested: (1) the superstimulatory response is related to the intrinsic number of follicles recruited into a follicular wave; and (2) the number of follicles recruited into a wave is correlated to the number of follicles recruited into the successive wave. A positive correlation between the number of small follicles at the time of wave emergence and the superstimulatory response may form the basis of a simple ultrasound-based test for reliably predicting the superstimulatory response of individual cows. Such a test will permit selection of donor cows for optimal response, estimation of the superstimulatory response of individual donors, and estimation of the expected number of embryo recipients required.

2. Materials and methods

2.1. Animals and treatments

Lactating cross-bred Hereford cows, 2–8 year of age and 35 days postpartum ($n = 141$), were used during May and June. The ovaries of each cow were examined once (20–30 cows per day for 5 consecutive days) using transrectal ultrasonography (Aloka SSD 900 echocamera with a 7.5 MHz linear-array side-fire transducer, Instruments for Science and Medicine, Vancouver, BC, Canada) to record the number of follicles present in different size categories. This examination provided data on follicle numbers at random stages of follicular wave development. All ultrasound examinations and follicle counts were carried out by the same person. Immediately after ultrasound examination, cows were given 2.5 mg of estradiol-17β and 50 mg of progesterone (E/P; Sigma Catalog # E-8875 and P-0130, respectively, Sigma-Aldrich Co. Canada Ltd., Oakville, Ont., Canada) in 2 ml canola oil (i.m.) to induce follicular wave synchronization (first synchronization). The emergence of a new follicular wave was expected 4 days later [32]. A second ultrasound examination was done 5 days after E/P treatment to again record the number of follicles in the different size categories (wave emergence after first synchronization).

Two subsets of cows (expected high- and low-responders) were selected from the herd of 141 based on the numbers of ≥2 (total), 2–3, and 4–6 mm follicles counted on the day of wave emergence after the first synchronization. Cows were ranked in descending order according to the number of follicles (both ovaries combined) in each size category (i.e., total number of follicles, 2–3 mm follicles, and 4–6 mm follicles). To form high- and low-end groups, cows that ranked in the upper and lower 10% of total follicle numbers, and those in the upper and lower 5% of the other two size categories were selected (maximum $n = 28$ per group). Of the three rankings, 12 cows appeared in more than one selection list for the high-end group and eight cows appeared in more than one selection list for the low-end group. Therefore, the final number of cows was 16 for the high-end group and 20 for the low-end group.

Selected cows were given superstimulatory treatments after synchronization of the succeeding follicular wave (second synchronization). The second wave synchronization was done by transvaginal ultrasound-guided follicle ablation 9–11 days after E/P treatment; i.e., approximately 5–7 days after emergence of the previous wave. Ablation was accomplished by aspirating the contents of all follicles ≥6 mm in both ovaries using a 5-MHz end-fire convex-array transducer (Aloka SSD 500) fitted to the end of a custom-built

plastic extension enclosing a needle guide and a 19-gauge 50-cm long needle [39]. The follicle ablation technique was used to minimize the interval to next wave emergence and to avoid erroneous counting of large follicles of the previous wave during assessment of the superstimulatory response. Cows were examined by transrectal ultrasonography 24 h after ablation (i.e., the expected time of wave emergence) [39] to again record the number of follicles in different size categories (wave emergence after second synchronization).

Cows were divided into four replicates and superstimulatory treatment was initiated in each replicate on successive days (i.e., $n = 4$ high-end cows and $n = 5$ low-end cows per day) beginning 36 h after follicle ablation. Superstimulatory treatment consisted of a total dose of 200 mg FSH (Folltropin, Bioniche Animal Health Canada Inc., Belleville, Ont., Canada) divided bid over 3 days (i.e., six i.m. injections of 33 mg FSH). Half of the recommended dose of FSH was used to avoid excessive stimulation and to permit a more accurate count of developing follicles by ultrasonography [27]. The superstimulatory response was assessed by transrectal ultrasound examination on the day after the last FSH treatment (i.e., 5 days after follicle ablation). The number of follicles in the 5–7, ≥8, and ≥5 mm (all) categories were recorded. The number of ovulations was not included as an end point in the present study because follicles were aspirated prior to ovulation to collect oocytes for the purposes of a separate study.

2.2. Plasma FSH at wave emergence

Plasma samples were obtained by jugular venipuncture 0, 8, 16, 24, and 32 h after follicle ablation (i.e., before initiation of ovarian superstimulatory treatment) to compare the endogenous rise in circulating concentrations of FSH at wave emergence in the high- and low-end groups. FSH levels were measured by radioimmunoassay [40,41] in a single batch, with intra-assay coefficients of variation of 5.9 and 6.1 for reference plasma samples of 1.8 and 4.0 ng/ml, respectively, and with a minimum detectable limit of 0.13 ng/ml.

2.3. Statistical analyses

The number of follicles in the ≥2 (total), 2–3, and 4–6 mm categories on the random day of the follicular wave and on the day of wave emergence after the first and second synchronizations were compared by two-tailed Student's *t*-tests (comparisons between two groups) or by ANOVA (more than two groups). Repeated measures data for FSH was analyzed by Proc Mixed (SAS system for Windows version 8.02, SAS Institute Inc., Cary, NC, USA) using autoregressive covariance matrix. If the ANOVA or Proc. Mixed indicated a significant difference ($P \leq 0.05$), post hoc comparisons were made using Tukey's adjusted least significant difference. Pearson's correlation coefficient (r) and significance level (P-value) were used to examine follicle–number relationships between two time periods. Spearman's correlation coefficient (ρ) and significance level (P-value) were used to examine the relationship between the rank orders of high- and low-end cows based on follicle numbers in different size categories. Best-subset linear regression analysis (Minitab version 13, Minitab Inc., State College, PA, USA) was performed to identify best-fitting predictors of the number of 5–7, >8, and >5 mm (all) follicles after ovarian superstimulation. The method allows determination of models with as few predictors as

possible. The full model contained 9 predictors (number of ≥2, 2–3, and 4–6 mm follicles at wave emergence after first and second follicular wave synchronizations, and on random day of the follicle wave). Analyses were performed to select the best and second-best models containing one predictor, two predictors, and three predictors. To obtain the most reliable prediction of ovarian superstimulatory response based on a single ultrasound examination (highest r^2 and r^2-adjusted values for multiple regression equation), follicle number data at wave emergence after second synchronization were investigated further by including linear (number), quadratic (number^2) and cubic (number^3) terms for the number of 2–3, 4–6, and ≥2 mm (total) follicles (nine predictors) in best-subset regression analyses.

3. Results

3.1. Selection of cows for ovarian superstimulation and number of follicles at wave emergence

The mean (±S.D.) number of follicles ≥2 mm detected per cow at wave emergence after the first synchronization was 30.7 ± 9.0, and was normally distributed among cows in the herd (Anderson–Darling normality test, $P < 0.01$; $n = 141$; Fig. 1). Follicles (mean ± S.E.M.) in the 2–3 mm (13.8 ± 0.6) and 4–6 mm (14.6 ± 0.6) categories constituted 45 and 48% (i.e., 93%) of the total number of follicles ≥2 mm detected in the ovaries.

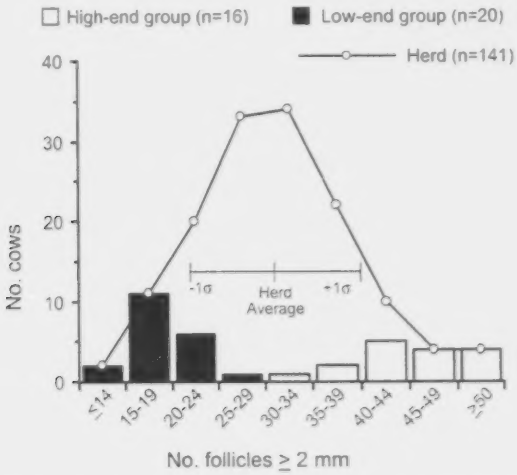

Fig. 1. Frequency distribution of cows in the whole herd (line), and in the low- (black bars) and high-end groups (white bars) based on the number of ≥2 mm follicle in both ovaries at wave emergence after first synchronization. Low- ($n = 20$) and high-end ($n = 16$) cows were selected from the herd ($n = 141$) for ovarian superstimulation to include top and bottom 10% of herd ranked by number of follicles at wave emergence (see Section 2 for details). Twenty-eight out of 36 selected cows were outside the herd mean ± first standard deviation (horizontal line).

Table 1
The number of follicles (mean ± S.E.M.) at the time of wave emergence and the relationship (ratio and Pearson's correlation, *r*) between the number of 2–3 and 4–6 mm follicles in cows selected to be among the upper and lower 10% of a herd based the number of follicles detected by ultrasonography

	No. of follicles			2–3 mm vs. 4–6 mm	
	≥2 mm	2–3 mm	4–6 mm	Ratio	*r* (*P* value)[f]
High-end group on day of wave emergence ($n = 16$)					
First synchronization	46.2 ± 2.1[ab]	21.6 ± 2.8[ab]	23.1 ± 2.4[a]	0.94	−0.66 (<0.01)
Second synchronization	51.1 ± 3.1[a]	32.8 ± 2.8[c]	17.6 ± 2.6[ab]	1.86	−0.17 (0.54)
Low-end group on day of wave emergence ($n = 20$)					
First synchronization	18.6 ± 1.3[e]	9.5 ± 1.4[d]	7.0 ± 0.9[e]	1.35	−0.55 (0.01)
Second synchronization	27.6 ± 2.7[d]	15.8 ± 2.1[adef]	11.4 ± 1.7[b]	1.39	0.02 (0.94)
Combined high- and low-end groups on day of wave emergence ($n = 36$)					
First synchronization	31.2 ± 2.6[de]	15.0 ± 1.8[ade]	14.4 ± 1.8[b]	1.05	0.08 (0.66)
Second synchronization	38.0 ± 2.9[be]	23.4 ± 2.2[bcf]	14.2 ± 1.6[b]	1.61	0.16 (0.36)
Whole herd on day of wave emergence ($n = 141$)					
First synchronization	30.7 ± 0.8[d]	13.8 ± 0.6[e]	14.6 ± 0.6[b]	0.95	0.09 (0.25)

Values within columns with different letters (a, b, c, d, e, f) in superscript differ ($P < 0.05$).

respectively (Table 1). Follicle numbers for 28 of the 36 cows selected in the high- and low-end groups were outside the herd mean ± first standard deviation (Fig. 1). The ovaries of cows in the high-end group contained more ($P < 0.001$) follicles ≥2 mm (total) at wave emergence than those of cows in the low-end group (Table 1; Fig. 2) after both the first and second follicular wave synchronizations. The total number of follicles in the low-end group was higher ($P = 0.003$) after ablation (second wave synchronization) than after E/P treatment (first wave synchronization), but no such difference was detected in the high-end group ($P = 0.22$). The magnitude of the difference between the high- and low-end groups in the number of follicles detected at wave emergence was similar between the first and second synchronizations (Fig. 2). The average number of follicles in each category at wave emergence for the high- and low-end groups combined ($n = 36$) was similar to that for the whole herd ($n = 141$), but was greater and lesser, respectively ($P < 0.01$), for the high- and low-end groups compared to the whole herd (Table 1).

3.2. Correlation between follicle numbers in the 2–3 and 4–6 mm categories

The numbers of 2–3 and 4–6 mm follicles were not correlated ($P = 0.25$) in cows in the whole herd ($n = 141$), nor in selected cows ($n = 36$), except at wave emergence after the first synchronization ($P = 0.01$). The ratio of the number of 2–3 mm versus 4–6 mm follicles ranged from 0.94 to 1.86 (Table 1).

3.3. Correlation between follicle numbers in successive waves

High values for Pearson's and Spearman's correlation coefficients were detected between successive waves (i.e., at wave emergence after first and second synchronizations)

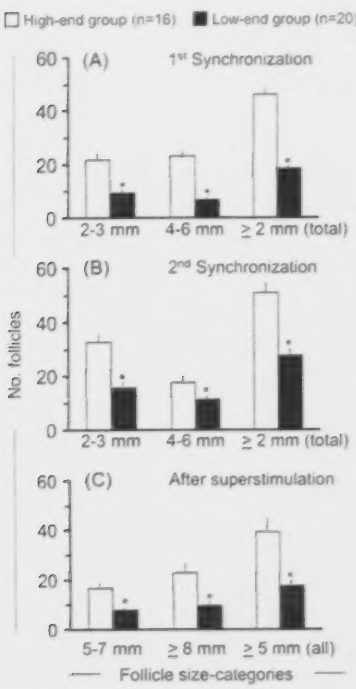

Fig. 2. Number of follicles detected at the emergence of the first (A), second (B) synchronized waves, and at the end of ovarian superstimulatory treatment (C) in the high-end group (white bars) and low-end group (black bars) chosen from a herd of 141 cows based on follicle numbers. A difference ($P < 0.05$) between high- and low-end groups is indicated by an asterisk (*).

based on the number of follicles in the ≥2 mm ($r = 0.77$, $\rho = 0.71$) and 2–3 mm ($r = 0.71$, $\rho = 0.71$) categories (Table 2). Ranking of cows based on follicle numbers on random days of the follicular wave, however, was not correlated with numbers detected after the first and second wave synchronizations. Correlation between the number of follicles detected on random days of the follicular wave versus the day of wave emergence after the first and second synchronizations were either nonsignificant ($P > 0.17$ for 4–6 mm follicles) or values were numerically lower (for 2–3 and ≥2 mm) than those between the first and second synchronizations (Table 2).

3.4. Superstimulatory response of high- and low-end groups

A greater number of 5–7, ≥8, and ≥5 mm (all) follicles was detected on the day after the last FSH treatment in the high-end group than in the low-end group (16.6 ± 2.2 versus 8.1 ± 0.9, 22.7 ± 4.1 versus 9.7 ± 1.6, and 39.4 ± 5.4 versus 17.8 ± 2.0, respectively; Fig. 2). The numbers of follicles in the 5–7 mm and ≥8 mm categories at the end of ovarian superstimulation were positively correlated ($r = 0.55$, $P = 0.001$).

Table 2
Relationship between the numbers of follicles in different size categories in successive waves in cows (high- and low-end groups combined; $n = 36$)

		r	ρ
Correlation between follicle numbers on random days vs. the day of wave emergence			
2–3 mm	First synchronization	0.61 (<0.01)	0.17 (0.33)
	Second synchronization	0.55 (<0.01)	0.11 (0.53)
4–6 mm	First synchronization	0.18 (0.28)	0.17 (0.32)
	Second synchronization	0.23 (0.17)	−0.09 (0.62)
≥2 mm	First synchronization	0.59 (<0.01)	0.23 (0.17)
	Second synchronization	0.63 (<0.01)	0.21 (0.22)
Correlation between follicle numbers on the day of wave emergence after the first vs. second synchronization (successive waves)			
2–3 mm		0.71 (<0.01)	0.71 (<0.01)
4–6 mm		0.45 (<0.01)	0.49 (<0.01)
≥2 mm		0.77 (<0.01)	0.71 (<0.01)

For Pearson's correlation coefficient (r), values for number of follicles were compared and for Spearman's correlation coefficient (ρ), ranks of the cows based on follicle numbers were compared. P-values for correlation coefficients are given in parentheses.

3.5. Plasma FSH concentrations at wave emergence

A peak (time effect $P < 0.0001$) in plasma concentrations of FSH was detected (Fig. 3A) 16–24 h after follicular ablation in both low- and high-end groups (27 out of 36 cows). The interval between follicular ablation and the FSH peak was not different between high- and low-end groups (18.5 ± 2.2 versus 21.2 ± 1.7, $P = 0.32$); however, the median value was 16 h for the high-end group and 24 h for the low-end group. When data were centralized to the FSH peak (Fig. 3B), the low-end group had a higher peak value ($P = 0.043$) and a steeper rise and fall (time-by-group interaction, $P = 0.03$) in plasma FSH concentrations than the high-end group.

3.6. Correlation between follicle numbers at wave emergence and the superstimulatory response

The number of 5–7, ≥8, and ≥5 mm (all) follicles and the ranking of cows at the end of superstimulation were strongly correlated with the number of follicles and ranking at the time of wave emergence (first and second synchronizations), but not to follicle numbers and ranking on random days of the follicular wave (Table 3). The strongest correlations to the superstimulatory response were the total number of follicles (≥2 mm) at wave emergence after the second synchronization (same wave) and first synchronization (preceding wave). The numbers of follicles in the 2–3 and 4–6 mm categories prior to superstimulation were also highly correlated with the superstimulatory response. Both Pearson's correlation and Spearman's rank correlation values between the total number of

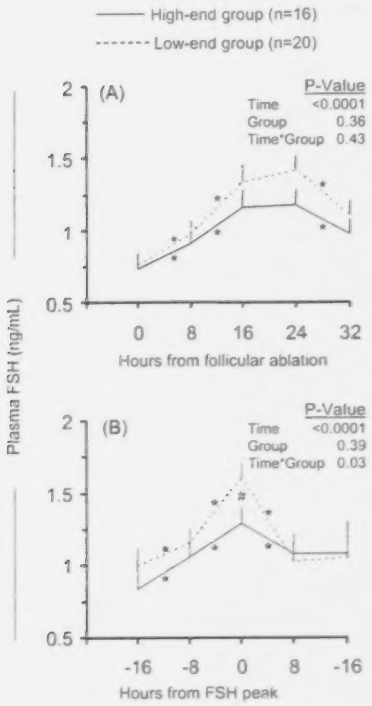

Fig. 3. Plasma FSH concentration (mean ± S.E.M.) at wave emergence after the second synchronization (follicular ablation) in high- (solid line) and low-end (broken line) groups chosen from a herd of 141 cows based on follicle numbers. Data are illustrated as hr after follicular ablation (A) or centralized to the peak in FSH of individual cows (B). Within groups, difference ($^{*}P < 0.05$) between adjacent values. Peak values differed ($^{\#}P = 0.043$) between groups.

follicles (≥2 mm) at wave emergence and the number of 5–7 mm follicles after ovarian superstimulation were numerically higher than any other correlation.

3.7. Best predictors of superstimulatory response

Best-subset linear regression analysis (Table 4) was performed to identify best-fitting predictors of the number of 5–7, ≥8, and ≥5 mm (all) follicles after ovarian superstimulation. The total number of follicles (≥2 mm) at wave emergence after the second and first synchronizations were the best and second-best predictors of the number of 5–7 and ≥5 mm (all) follicles after superstimulation, respectively. The total number of follicles (≥2 mm) at wave emergence after the first synchronization was the best predictor of the number of follicles (≥8 mm) after superstimulation.

To predict the ranking of cows according to the number of 5–7, ≥8, and ≥5 mm (all) follicles after superstimulation, similar best-subset regression analyses were performed on

Table 3
Relationship between the number of follicles detected before vs. after ovarian superstimulation ($n = 36$)

Correlation between no. of follicles		r	ρ
Random day of wave	After superstimulation		
2–3 mm	5–7 mm	0.37 (0.03)	0.36 (0.03)
	≥8 mm	0.16 (0.36)	0.01 (0.95)
	≥5 mm (all)	0.26 (0.12)	0.19 (0.26)
4–6 mm	5–7 mm	0.24 (0.16)	−0.002 (0.99)
	≥8 mm	0.12 (0.49)	0.16 (0.35)
	≥5 mm (all)	0.18 (0.29)	0.11 (0.53)
≥2 mm	5–7 mm	0.48 (<0.01)	0.40 (0.02)
	≥8 mm	0.19 (0.24)	0.09 (0.58)
	≥5 mm (all)	0.34 (0.04)	0.29 (0.09)
Wave emergence after first synchronization			
2–3 mm	5–7 mm	0.46 (<0.01)	0.59 (<0.01)
	≥8 mm	0.25 (0.14)	0.29 (<0.01)
	≥5 mm	0.37 (0.03)	0.49 (<0.01)
4–6 mm	5–7 mm	0.47 (<0.01)	0.59 (<0.01)
	≥8 mm	0.53 (<0.01)	0.29 (0.09)
	≥5 mm (all)	0.57 (<0.01)	0.49 (<0.01)
≥2 mm	5–7 mm	0.62 (<0.01)	0.57 (<0.01)
	≥8 mm	0.54 (<0.01)	0.29 (0.09)
	≥5 mm (all)	0.64 (<0.01)	0.49 (<0.01)
Wave emergence after second synchronization			
2–3 mm	5–7 mm	0.62 (<0.01)	0.60 (<0.01)
	≥8 mm	0.45 (<0.01)	0.40 (0.02)
	≥5 mm	0.58 (<0.01)	0.57 (<0.01)
4–6 mm	5–7 mm	0.43 (<0.01)	0.27 (0.12)
	≥8 mm	0.29 (0.09)	0.21 (0.22)
	≥5 mm (all)	0.38 (0.02)	0.34 (0.04)
≥2 mm	5–7 mm	0.71 (<0.01)	0.58 (<0.01)
	≥8 mm	0.50 (<0.01)	0.29 (0.09)
	≥5 mm (all)	0.65 (<0.01)	0.49 (<0.01)

For Pearson's correlation coefficient (r), values for number of follicles were compared and for Spearman's correlation coefficient (ρ), ranks of the cows based on follicle numbers were compared. P-values for correlation coefficients are given in parentheses.

cow rankings (Table 5). Ranking according to the total number of follicles (≥2 mm) at wave emergence after the second synchronization was the best predictor of ranking according to ≥5 and ≥8 mm follicles after superstimulation. Ranking according to the number of 2–3 mm follicles at wave emergence after second synchronization best predicted the ranking according to 5–7 mm follicles after superstimulation.

Table 4
Best predictor/s of superstimulatory response were determined by best-sub

		r^2-value	Day of wave emergenc second synchronizatio	
			≥2 mm	2–3 mm
Response variable: number of 5–7 mm follicles after superstimulation				
One predictor	Best	49.8	X	
	Second best	38.9		
Two predictors	Best	51.5	X	
	Second best	51.4	X	
Three predictors	Best	51.9	X	
	Second best	51.8	X	
Full model		53.3	X	X
Response variable: number of ≥8 mm follicles after superstimulation				
One predictor	Best	28.7		
	Second best	28.0		
Two predictors	Best	32.6		
	Second best	32.6		X
Three predictors	Best	34.8	X	
	Second best	34.2		
Full model		36.3	X	X
Response variable: number of ≥5 mm follicles after superstimulation				
One predictor	Best	42.1	X	
	Second best	40.8		
Two predictors	Best	46.8	X	
	Second best	46.1	X	
Three predictors	Best	48.6	X	
	Second best	48.0	X	
Full model		49.5	X	X

"X" in a cell indicated that the predictor was selected. Analysis was performed to
comparison

sets linear regression analysis. Based on r^2-value of fit, best and second-best model are reported

ce after n		Day of wave emergence after first synchronization			Random day of wave		
n	4–6 mm	≥2 mm	2–3 mm	4–6 mm	≥2 mm	2–3 mm	4–6 mm
		X					
			X				
		X					
			X	X			
		X		X			
	X	X	X	X	X	X	X
		X					
				X			
			X	X			
				X			
		X			X		
	X	X			X		
	X	X	X	X	X	X	X
		X					
		X					
				X			
		X			X		
			X	X			
	X	X	X	X	X	X	X

include one, two, and three predictor/s. Full model (including all nine predictors) r^2 values are provided for

Table 5
Best predictor/s of ranking of cows based on superstimulatory response v

		r^2-value	Ranking at wave emerg second synchronization	
			≥2 mm	2–3 mm
Response variable: ranking of cows based on number of 5–7 mm follicles after sup				
One predictor	Best	36.2		X
	Second best	34.7		X
Two predictors	Best	46.4		X
	Second best	45.4		X
Three predictors	Best	49.1		X
	Second best	49.0		X
Full model		51.7	X	X
Response variable: ranking of cows based on number of ≥8 mm follicles after sup				
One predictor	Best	16.2	X	
	Second best	15.7		X
Two predictors	Best	18.5	X	
	Second best	17.9		X
Three predictors	Best	20.4		X
	Second best	19.1	X	X
Full model		26.2	X	X
Response variable: ranking of cows based on number of ≥5 mm follicles after sup				
One predictor	Best	34.8	X	
	Second best	32.8		X
Two predictors	Best	39.0		X
	Second best	37.5	X	
Three predictors	Best	42.6	X	X
	Second best	41.2		X
Full model		46.2	X	X

Based on the r^2 value of fit, the best and second-best models are presented. An "X" predictor/s. The full model (including all nine predictors) values are provided for

were determined by best-subsets linear regression analysis

ence after (n = 36)	Ranking at wave emergence after first synchronization (n = 36)			Ranking on random day of wave (n = 141)		
4–6 mm	≥2 mm	2–3 mm	4–6 mm	≥2 mm	2–3 mm	4–6 mm
erstimulation						
			X			
				X		
		X		X		
			X	X		
X	X	X	X	X	X	X
erstimulation						
						X
						X
X						X
						X
X	X	X	X	X	X	X
erstimulation						
X						
				X		
X						
X				X		
X	X	X	X	X	X	X

" in a cell indicates that the predictor was selected. Analyses were performed to include one, two, or three comparison

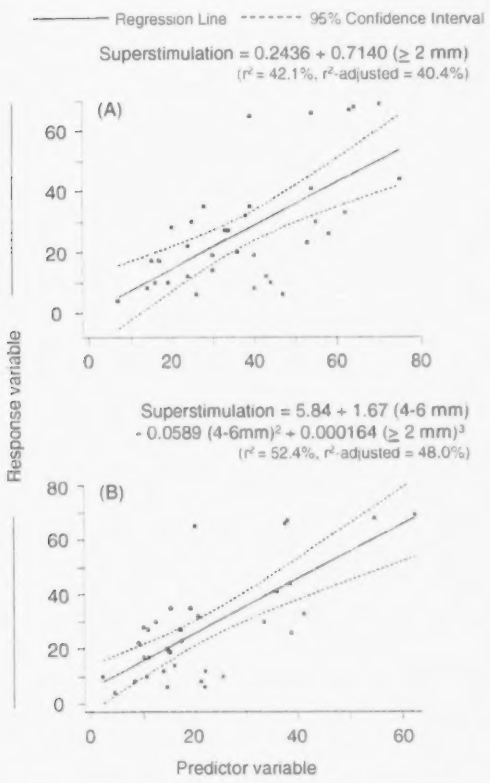
Regression Line
95% Confidence Interval
Superstimulation = 0.2436 + 0.7140 (≥ 2 mm)
(r² = 42.1%, r²-adjusted = 40.4%)
(A)
Superstimulation = 5.84 + 1.67 (4-6 mm)
- 0.0589 (4-6mm)² + 0.000164 (≥ 2 mm)³
(r² = 52.4%, r²-adjusted = 48.0%)
(B)
Response variable
Predictor variable

Fig. 4. Regression analysis for predicting the ovarian superstimulatory response in cattle based on the number of follicles detected at the time of wave emergence (i.e., after the second synchronization). Dots represent observed values for individual cows ($n = 36$). (A) A simple linear regression between the number of follicles ≥5 mm after ovarian superstimulation (response variable) and the number of follicles ≥2 mm at wave emergence (predictor variable). (B) A multiple polynomial regression between the number of follicles ≥5 mm after ovarian superstimulation (response variable) and the number of ≥2 and 4–6 mm follicles at wave emergence (predictor variable).

3.8. Regression equations to predict superstimulatory response

Based on best-subset linear regression analyses, the total number of follicles at wave emergence of the same wave returned the highest r^2-values (Table 4). Fig. 4A illustrates the simple regression between the number of total follicles (≥2 mm) detected at wave emergence after the second synchronization and the number of follicles ≥5 mm after ovarian superstimulation. The number of follicles ≥5 mm detected after superstimulation was approximately 71% of the number of follicles ≥2 mm detected at wave emergence after the second synchronization. The most reliable predictor (i.e., highest r^2 and r^2-adjusted values) of the superstimulatory response was expressed by the cubic regression equation illustrated in Fig. 4B.

4. Discussion

The results supported the hypotheses that the number of follicles recruited into a wave is correlated to the number of follicles recruited into the successive wave, and the superstimulatory response is related to the intrinsic number of follicles recruited into a follicular wave. The number of follicles recruited into two successive waves and the ranks of cows based on follicle numbers were highly repeatable. A stronger correlation existed between the number of follicles at the time of emergence of two successive waves than between random days of wave and day of emergence of the next wave. It was not surprising that the number of 4–6 mm follicles present at wave emergence was not correlated to the number of follicles present on random days of the wave, since the number of follicles within a given size category changes over the course of a follicular wave [42,43]. However, the number of 2–3 mm follicles at random days of the wave was correlated to the number of 2–3 mm follicles at wave emergence, although not as strongly as numbers at the day of emergence of two successive waves. This may be explained by a smaller magnitude of change in the number of small follicles than in medium follicles over different days of wave. Overall, ultrasound examination on random days of a follicular wave is less accurate for predicting follicle numbers or donor ranking at wave emergence or after superstimulation than assessment at the time of wave emergence.

Selection of the top and bottom 10% of the herd based on the number of follicles at wave emergence was validated by the results of the present study. The superstimulatory response was twice as high in the top ranked cows than in the bottom ranked cows. In general, the total number of follicles $\geq$2 mm was a better predictor of superstimulatory response than the number of 2–3 or 4–6 mm follicles alone. A cubic multiple regression equation based on the number of follicles present at wave emergence (Fig. 4) provided a reliable prediction of the superstimulatory response, but for practical purposes, one can expect the number of follicles $\geq$5 mm after ovarian superstimulation to be approximately 71% of the number of follicles $\geq$2 mm at the time of wave emergence. In the present study, follicles were aspirated at the end of superstimulatory treatment; therefore, data on the number of ovulations and embryos produced was not available. Further studies are required to address these end points.

That the FSH peak was higher in low-end group than in the high-end group was an unexpected and an intriguing observation. As expected, FSH concentrations were not different between low- and high-end groups at the time of follicular ablation (second follicular wave synchronization) because all cows were between Days 5 and 7 of the previous wave (expected nadir in FSH). Perhaps, relatively lower number of small follicles in the low-end group at wave emergence permitted a more rapid rise in FSH. The question remains: Why do low-end cows have fewer follicles in each wave? Two possible explanations may be that there are inherently fewer follicles within the growing pool, or small follicles are less responsive to FSH in the low-end group.

The results of this study provide the basis of a simple ultrasound test to predict the superstimulatory response in cattle: (Step 1) Induce a new wave by follicular ablation or E/P treatment. (Step 2) Count the number of follicles $\geq$2 mm in both ovaries by ultrasonography on the day of wave emergence (1 day after ablation or 4 days after E/P

treatment). (Step 3) Select donors based on the desired response and recipient availability. (Step 4) Start superstimulatory treatment at wave emergence (i.e., immediately on the day of follicle count, or at a future-scheduled wave emergence). Further validation of the test is required to: (i) determine the sensitivity and specificity; and (ii) quantify the correlation between follicle numbers at wave emergence, ovulations at end of superstimulation, and the number of transferable embryos recovered.

In conclusion, a very strong relationship existed between numbers of follicles $\geq$2 mm present at the time of emergence of two successive waves, suggesting that the expected number of follicles at next wave emergence can be predicted with a high level of reliability (Pearson correlation coefficient = 0.77, $P = 0.0001$). The number of follicles at the end of superstimulatory treatment was also correlated to, and can be predicted by, the number of follicles recruited into the same or preceding wave. Practitioners can expect the number of follicles $\geq$5 mm after ovarian superstimulation to be approximately 71% of the number of follicles $\geq$2 mm at the time of wave emergence. Based on the results of this study, a simple ultrasound test has been developed to predict the superstimulatory response in cattle. This simple test will permit: (i) a realistic expectation of donor response; (ii) selection of donors expected to provide a greater superstimulatory response (for oocyte retrieval or embryo transfer); and (iii) anticipation of embryo production needs (i.e., recipients for embryo transfer; laboratory supplies for IVF).

Acknowledgements

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References

[1] Looney CR. Superovulation in beef females. In: Proceedings of 5th Annual Convention of AETA, Fort Worth, Texas; 1986. p. 16–29.

[2] Lerner SP, Thayne WV, Baker RD, Henschen T, Meredith S, Inskeep EK, et al. Age, dose of FSH and other factors affecting superovulation in Holstein cows. J Anim Sci 1986;63:176–83.

[3] Tonhati H, Lobo RB, Oliveira HN. Repeatability and heritability of response to superovulation in Holstein cows. Theriogenology 1999;51:1151–6.

[4] Keller DS, Teepker G. Effect of variability in response to superovulation on donor cow selection differentials in nucleus breeding schemes. J Dairy Sci 1990;73:549–54.

[5] Kafi M, McGowan MR. Factors associated with variation in the superovulatory response of cattle. Anim Reprod Sci 1997;48:137–57.

[6] Monniaux D, Chupin D, Saumande J. Superovulatory responses of cattle ovaries of cows. Theriogenology 1983;19:55–81.

[7] van de Leemput EE, Vos PL, Hyttel P, van den Hurk R, Bevers MM, van der Weijden GC, et al. Effects of brief postponement of the preovulatory LH surge on ovulation rates and embryo formation in eCG/prostaglandin-treated heifers. Theriogenology 2001;55:573–92.

[8] Gonzalez A, Wang H, Carruthers TD, Murphy BD, Mapletoft RJ. Superovulation in the cow with pregnant mare serum gonadotrophin: effects of dose and antipregnant mare serum gonadotrophin serum. Can Vet J 1994;35:158–62.
[9] Vos PL, van der Schans A, de Wit AA, Bevers MM, Willemse AH, Dieleman SJ. Effects of neutralization of pregnant mares' serum gonadotrophin (PMSG) shortly before or at the preovulatory LH surge in PMSG-superovulated heifers on follicular function and development. J Reprod Fertil 1994;100:387–93.
[10] Armstrong DT, Opavsky MA. Biological characteristics of a pituitary FSH preparation with reduced LH activity. Theriogenology 1986;25:135 [abstract].
[11] Looney CR, Bondioli KR, Hill KG, Massey JM. Superovulation of donor cows with bovine follicle-stimulating hormone (bFSH) produced by recombinant DNA technology. Theriogenology 1988;29:271.
[12] Gonzalez A, Lussier JG, Carruthers TD, Murphy BD, Mapletoft RJ. Superovulation of beef heifers with folltropin. A new FSH preparation containing low LH activity. Theriogenology 1990;33:519–29.
[13] Willmott N, Saunders J, Bo GA, Palasz A, Pierson RA, Mapletoft RJ. The effect of FSH/LH ratio in pituitary extracts on superovulatory response in the cow. Theriogenology 1990;33:347.
[14] Tribulo H, Bo GA, Jofre F, Carcedo J, Alonso A, Mapletoft RJ. The effect of LH concentration in a pituitary extract and season on superovulatory response of Bos indicus heifers. Theriogenology 1991; 35:286.
[15] Alkemade SJ, Murphy BD, Mapletoft RJ. Superovulation in the cow: effects of biological activity of gonadotropins. In: Proceedings of 12th Annual Convention of AETA, Portland, Maine; 1993.
[16] Bo GA, Hockley DK, Nasser LF, Mapletoft RJ. Superovulatory response to a single subcutaneous injection of Folltropin-V in beef cattle. Theriogenology 1994;42:963–75.
[17] Mapletoft RJ, Steward KB, Adams GP. Recent advances in the superovulation in cattle. Reprod Nutr Dev 2002;42:601–11.
[18] Adams GP. Control of ovarian follicular wave dynamics in mature and prepubertal cattle for synchronization and superstimulation. In: Proceedings of the XX Congress of the World Association for Buiatrics, Sydney, Australia; 1998. p. 595–605.
[19] Merton JS, de Roos AP, Mullaart E, de Ruigh L, Kaal L, Vos PL, et al. Factors affecting oocyte quality and quantity in commercial application of embryo technologies in the cattle breeding industry. Theriogenology 2003;59:651–74.
[20] Kim IH, Son DS, Yeon SH, Choi SH, Park SB, Ryu IS, et al. Effect of dominant follicle removal before superstimulation on follicular growth, ovulation and embryo production in Holstein cows. Theriogenology 2001;55:937–45.
[21] Guilbault LA, Grasso F, Lussier JG, Rouillier P, Matton P. Decreased superovulatory responses in heifers superovulated in the presence of a dominant follicle. J Reprod Fertil 1991;91:81–9.
[22] Diaz T, Pancarci SM, Drost M, Schmitt EJ, Ambrose JD, Fredriksson WE, et al. Effects of the persistent dominant follicle on the ability of follicle stimulating hormone to induce follicle development and ovulatory responses. J Dairy Sci 2001;84:88–99.
[23] Bungartz L, Niemann H. Assessment of the presence of a dominant follicle and selection of dairy cows suitable for superovulation by a single ultrasound examination. J Reprod Fertil 1994;101:583–91.
[24] Shaw DW, Good TE. Recovery rates and embryo quality following dominant follicle ablation in superovulated cattle. Theriogenology 2000;53:1521–8.
[25] Baracaldo MI, Martinez MF, Adams GP, Mapletoft RJ. Superovulatory response following transvaginal follicle ablation in cattle. Theriogenology 2000;53:1239–50.
[26] Adams GP. Control of ovarian follicular wave dynamics in cattle: implications for synchronization and superstimulation. Theriogenology 1994;41:19–24.
[27] Nasser LF, Adams GP, Bo GA, Mapletoft RJ. Ovarian superstimulatory response relative to follicular wave emergence in heifers. Theriogenology 1993;40:713–24.
[28] Adams GP, Nasser LF, Bo GA, Garcia A, Del-Campo MR, Mapletoft RJ. Superovulatory response of ovarian follicles of wave 1 versus wave 2 in heifers. Theriogenology 1994;42:1103–13.
[29] Bo GA, Adams GP, Pierson RA, Mapletoft RJ. Exogenous control of follicular wave emergence in cattle. Theriogenology 1995;43:31–40.
[30] Bo GA, Baruselli PS, Moreno D, Cutaia L, Caccia M, Tribulo R, et al. The control of follicular wave development for self-appointed embryo transfer programs in cattle. Theriogenology 2002;57:53–72.

[31] Bergfelt DR, Bo GA, Mapletoft RJ, Adams GP. Superovulatory response following ablation-induced follicular wave emergence at random stages of the oestrous cycle in cattle. Anim Reprod Sci 1997;49: 1–12.
[32] Mapletoft RJ, Martinez MF, Adams GP, Kastelic JP, Burnley C. The effect of estradiol preparation on follicular wave emergence and superovulatory response in norgestomet-implanted cattle. Theriogenology 1999;51:411.
[33] Adams GP, Matteri RL, Kastelic JP, Ko JCH, Ginther OJ. Association between surges of follicle-stimulating hormone and the emergence of follicular waves in heifers. J Reprod Fertil 1992;94:177–88.
[34] Adams GP, Kot K, Ginther OJ. Selection of a dominant follicle and suppression of follicular growth in heifers. Anim Reprod Sci 1993;30:259–71.
[35] Adams GP, Kot K, Smith CA, Ginther OJ. Effect of the dominant follicle on regression of its subordinates in heifers. Can J Anim Sci 1993;73:267–75.
[36] Singh J, Brogliatii GM, Christensen CR, Adams GP. Active immunization against follistatin and its effect on FSH, follicle development and superovulation in heifers. Theriogenology 1999;52:49–66.
[37] Cushman RA, DeSouza JC, Hedgpeth VS, Britt JH. Superovulatory response of one ovary is related to the micro- and macroscopic population of follicles in the contralateral ovary of the cow. Biol Reprod 1999; 60:349–54.
[38] Boni R, Roelofsen MWM, Pieterse MC, Kogut J, Kruip TAM. Follicular dynamics, repeatability and predictability of follicular recruitment in cows undergoing repeated follicular puncture. Theriogenology 1997;48:277–89.
[39] Bergfelt DR, Lightfoot KC, Adams GP. Ovarian synchronization following ultrasound-guided transvaginal follicle ablation in heifers. Theriogenology 1994;42:895–907.
[40] Rawlings NC, Jeffcoate IA, Reiger DL. The influence of estradiol17 and progesterone on peripheral serum concentrations of luteinizing hormone and follicles stimulating hormone on the ovariectomized ewe. Theriogenology 1984;22:473–88.
[41] Bo GA, Adams GP, Nasser LF, Pierson RA, Mapletoft RJ. Effect of estradiol valerate on ovarian follicles, emergence of follicular waves and circulating gonadotropins in heifers. Theriogenology 1993;40:225–37.
[42] Ginther OJ, Knopf L, Kastelic JP. Temporal associations among ovarian events in cattle during oestrous cycles with two and three follicular waves. J Reprod Fertil 1989;87:223–30.
[43] Pierson RA, Ginther OJ. Follicular populations during the estrous cycle in heifers I: influence of day. Anim Reprod Sci 1987;124:165–76.

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Promise of new imaging technologies for assessing ovarian function

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Abstract

Advancements in imaging technologies over the last two decades have ushered a quiet revolution in research approaches to the study of ovarian structure and function. The most significant changes in our understanding of the ovary have resulted from the use of ultrasonography which has enabled sequential analyses in live animals. Computer-assisted image analysis and mathematical modeling of the dynamic changes within the ovary has permitted exciting new avenues of research with readily quantifiable endpoints. Spectral, color-flow and power Doppler imaging now facilitate physiologic interpretations of vascular dynamics over time. Similarly, magnetic resonance imaging (MRI) is emerging as a research tool in ovarian imaging. New technologies, such as three-dimensional ultrasonography and MRI, ultrasound-based biomicroscopy and synchrotron-based techniques each have the potential to enhance our real-time picture of ovarian function to the near-cellular level. Collectively, information available in ultrasonography, MRI, computer-assisted image analysis and mathematical modeling heralds a new era in our understanding of the basic processes of female and male reproduction.

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Keywords: Follicle development; Folliculodynamics; Image analysis; MRI; Three-dimensional ultrasonography; Ultrasonography; Ultrasound

1. Introduction

Since the time of de Graaf, studies of follicle development involved data gathered from gross and histologic examination of excised ovaries (Jocelyn and Setchell, 1972). Even the most detailed studies of static specimens have excluded the fundamental processes of

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life and the mechanisms controlling them. Although knowledge gained from the study of tissue samples ex vivo has been considerable, a deeper understanding of structure–function relationships was obscured by the static nature of examination and considerable confusion about kinetics of ovarian follicle growth and development prevailed. During the 1980s a period of enlightenment in ovarian biology began with the introduction of real-time, B-mode ultrasonography. The ability to visualize changes in structure in a serial fashion, without interruption or distortion of function, has revitalized the study of ovarian biology. There has been a paradigm shift in our approaches to hypothesis testing and our understanding of folliculogenesis in domestic animals. It is now commonplace in ovarian research to include ultrasonographic imaging to monitor daily patterns of follicle growth and regression, and the ovarian response to experimental or therapeutic treatments.

Ultrasonography has heralded the way in medical imaging as a tool for ovarian research. Concurrently, continuous and rapid evolution of computer technology and medical electronics have created exciting new experimental and diagnostic capabilities in the analysis of visual data. The purpose of this manuscript is to provide an overview of imaging techniques that have been used in the study of ovarian function during the last two decades and to introduce emerging techniques that hold future promise. We begin with real-time B-mode ultrasonography and then venture through discussions of computer-assisted image analysis, three-dimensional imaging, vascular imaging, ultrasound biomicroscopy, magnetic resonance imaging (MRI), and synchrotron-based soft tissue X-ray imaging.

2. Ultrasonographic techniques in ovarian research

Gray-scale diagnostic ultrasonography is the most profound technological advance in the field of large-animal research and clinical reproduction since the introduction of transrectal palpation and radioimmunoassay of circulating hormones (Ginther, 1986). Nowhere have the effects of imaging been more evident than in the changes in our understanding of ovarian physiology. Changes in the number of peer-reviewed publications involving ovarian ultrasound imaging over the last 8 years illustrates widespread use of the technique for both research and clinical applications (Fig. 1). The technique has been used most extensively in humans and the bovine species, however, the emphasis of the ultrasound studies done in humans and animals is strikingly different. The distribution of studies in humans reflects a preoccupation with the use of ultrasonography as a tool for clinical diagnosis (i.e. detection and characterization of ovarian pathology), whereas the emphasis in animal studies is on ovarian physiology. More studies have been published on ovulation and follicular- and luteal-dynamics using the bovine model than in all other species combined. Interestingly, the use of ultrasonography has been reported frequently in both humans and animals for the study of follicular stimulation and ovulation induction, but use of the technique for oocyte retrieval has attracted more attention in animal studies even though transvaginal ultrasound-guided oocyte aspiration is the standard of practice in human assisted reproduction programs (Fig. 1).

One of the best examples of the impact of imaging on our understanding of reproductive function is that of ultrasonography and the proof of the wave-phenomenon of folliculogenesis. The wave theory of ovarian follicular development was originally proposed from

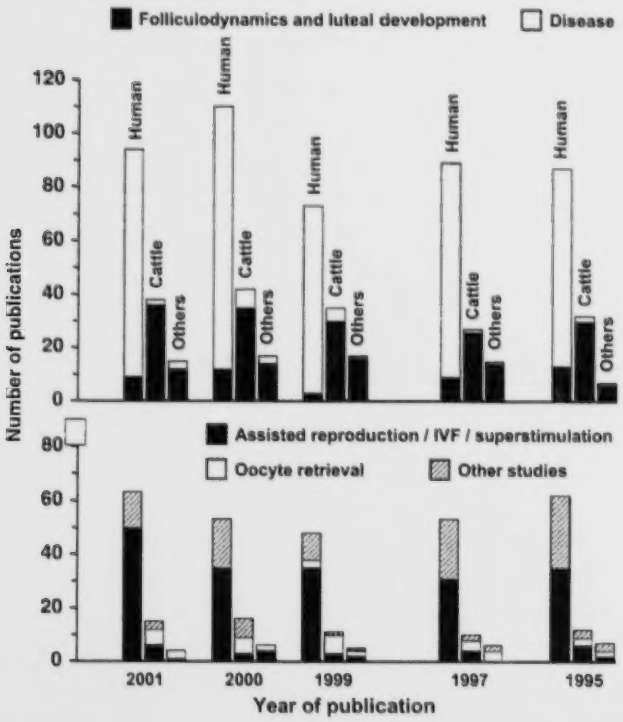

Fig. 1. Demographics of publications on ultrasound-based ovarian research in human, cattle, horse and other domestic species over the last 8 years. Data were obtained by searching for keywords—(ultrasound or ultrasonography) and (ovary or follicle or corpus luteum) in Agricola, Medline and CAB databases for human, cattle and other species (sheep, goat, mare, camlids, pig). Based on the title and year of publication, number of publications for each species were categorized into (i) folliculodynamics and luteal development, (ii) disease, (iii) assisted reproduction/IVF/superstimulation and (iv) other studies.

observations made in the bovine species (Rajakoski, 1960). It was based on gross and histologic evaluation of ovaries obtained from cows slaughtered on known days of the estrous cycle. From his detailed studies, Rajakoski concluded that two waves of antral follicle growth occurred during the estrous cycle. Others refuted the concept of wave phenomena and supported the notion that follicular growth is continuous and independent of the phases of the cycle (reviewed in Spicer and Echternkamp, 1986). Controversy surrounded the wave theory for nearly three decades, until the first ultrasonographic study of the bovine ovaries was reported in 1984 (Pierson and Ginther, 1984). Initial studies involved daily ultrasonography to monitor changes in follicle populations in different size categories (Pierson and Ginther, 1986, 1987a,b, 1988a). Results of these studies supported the wave theory but the population technique was not designed to monitor individually identified follicles; hence, the passage of an individual follicle from one size category to another could not be detected, and detailed characteristics of wave emergence and follicle selection were

inaccessible. Hallmark studies documenting the capability of monitoring individually identified follicles soon followed (Ginther et al., 1989a,b; Knopf et al., 1989; Pierson and Ginther, 1988b; Savio et al., 1988; Sirois and Fortune, 1988). This new capability created a flurry of interest and led to a series of advancements in our understanding of folliculogenesis and the development of methods to control ovarian function in cattle and other species (Adams, 1994, 1998, 1999; Fortune, 1993; Ginther et al., 1996, 2001). One of the most important advantages arising from the use of individual identity methods or the "non-identity" method (Ginther and Bergfelt, 1992) is the ability to normalize or centralize follicular and hormonal data about a specific point of reference (e.g. ovulation, wave emergence, follicle selection). This capability led to the development of a conceptual model of the generic follicular wave that provided an explanation of temporal relationships between endocrine and structural changes (Adams and Pierson, 1995). Incorporation of the wave concept has become an inherent part of experimental design in studies of ovarian function ranging from the molecular level to the whole-animal and herd level.

The bovine model, derived from studies in mature non-pregnant cows, has since been extended to other reproductive states and other species. The first study of follicular dynamics during pregnancy and the post-partum period in any species was in cattle (Pierson and Ginther, 1986; Ginther et al., 1989c; Savio et al., 1990). The first study of follicle dynamics during the neonatal and pre-pubertal periods in any species also was in cattle in 1993 (Adams et al., 1993; Evans et al., 1994a). Studies in other species soon followed, wave patterns of follicle development were documented in each: camelids in 1990 (Adams et al., 1990), horses in 1993 (Bergfelt and Ginther, 1993), sheep and goats in 1994 (Ginther and Kot, 1994; Ravindra et al., 1994), muskoxen—the first wild species in 1997 (Hoare et al., 1997), elk in 2001 (McCorkell et al., 2001, 2002), and most recently in humans (Baerwald et al., 2002a,b, 2003). Despite the wide range of reproductive patterns represented by these different species (i.e. polyestrous, seasonally polyestrous, single and multiple ovulators, spontaneous and induced ovulators, distinct luteal and follicular phases), the fundamental wave pattern of follicle development appears to be a widely preserved biologic phenomenon.

Ultrasound imaging technology has also revolutionized the study of the corpus luteum and has led to a greater understand of the interactions between luteogenesis and folliculogenesis. For instance, it is now clear that luteal production of progesterone has a suppressive effect on the growth of the dominant follicle of a wave (Adams et al., 1992; Stock and Fortune, 1993), and the magnitude of a luteal phase (progesterone concentration and duration) has an important impact on the interwave interval, follicular health, oocyte competence and subsequent luteal gland development. It has been shown that the luteal gland does not necessarily pass through a hemorrhagicum stage in mares (Pierson and Ginther, 1985; Ginther, 1995b). In addition, approximately 70% of bovine CL possess a fluid-filled cavity, the size of which changes during its lifespan (Kastelic et al., 1990b). In ultrasonographic studies, luteal tissue area (Kastelic et al., 1990a) and echotexture (Singh et al., 1997) was directly related to circulating and glandular concentrations of progesterone, and the presence of a fluid-filled cavity did not influence luteal function (Kastelic et al., 1990b). Ultrasonographic studies have resulted in the characterization of short luteal phases during the peri-pubertal period (Evans et al., 1994b), post-partum period (Rajamahendran and Taylor, 1990), and during seasonal recrudescence in sheep (Bartlewski et al., 1998) and elk (Adams, 1999). Luteal persistence has also been documented in relation to embryonic mortality (Kastelic and Ginther,

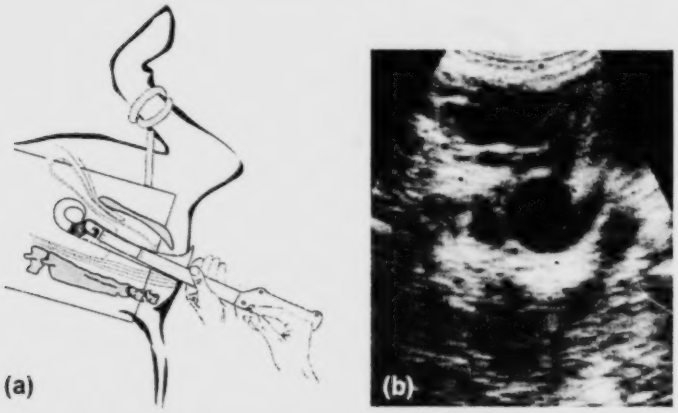

Fig. 2. Ultrasound-guided follicle aspiration technique for calves. (a) Demonstrates the topography of the genital organs and position of the transducer for transvaginal ultrasonography and oocyte aspiration in 6 week-old calves in dorsal recumbency. (b) Ultrasound image of an ovarian follicle during the aspiration procedure. The image of the needle entering the anechoic follicle is clearly observed (modified with permission from Brogliatti and Adams, 1996).

1989; Adams et al., 1991a), and ultrasonography has shed new light on the pathogenesis of ovarian irregularities, such as follicular and luteal cysts in cattle (Adams, 2000), hemorrhagic anovulatory follicles (HAF) in horses (Ginther, 1992) and llamas (Adams et al., 1991b), luteinized unruptured follicles (LUF) and polycystic ovarian syndrome (PCOS) in women (reviewed in Pierson and Chizen, 1994; Atiomo et al., 2000).

Ultrasound-guided techniques have provided the opportunity to collect oocytes transvaginally in mature (Pieterse et al., 1988) and prepubertal cattle (Brogliatti and Adams, 1996, Fig. 2), horses (Bracher et al., 1993; Cook et al., 1993) and in women (Wikland et al., 1983) for the purposes of in vitro embryo production. The technique has also led to novel studies involving follicular fluid sampling (Ginther et al., 1997), local or intra-follicular injections (Kot et al., 1995; Gastal et al., 1995), and gamete recovery and follicular transfer (Bergfelt et al., 1998; Hinrichs and DiGiorgio, 1991) to better understand the effects and capacity of individual follicles of a wave and their oocytes.

Ultrasonographic imaging has provided a vast improvement in our understanding of the wave pattern of folliculogenesis, but the question of why the wave pattern exists remains a mystery. We anticipate that ultrasonography and other imaging modalities will continue to stimulate scientific creativity to test hypotheses not previously conceivable.

3.[r] Computer-assisted image analysis

Computer-assisted analysis of image attributes is a natural extension of the technologic advances in diagnostic ultrasonography. This approach offers an exciting new method of determining follicle status at a single examination. In the present section, we provide an

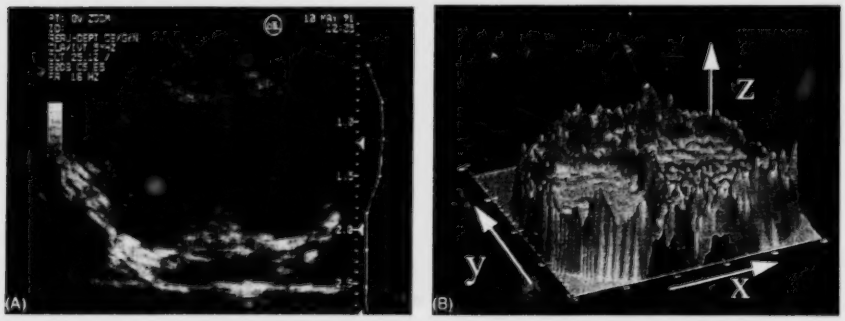

Fig. 3. (A) To illustrate the sensitivity of computer-assisted data analysis, an excised bovine ovary containing a preovulatory (left) and a regressing (right) follicle was scanned in a degassed water bath using a high resolution ultrasound instrument. The two large follicles imaged appear visually indistinguishable (B) Clear differences in the follicle walls and antra can be detected by computer-assisted analyses. In a three-dimensional representation of the brightness of the pixels comprising the ultrasound image, the echotexture of the antrum appears more heterogeneous and the pixels of the follicular wall are brighter and drop off more sharply in the follicle on the right. Image attributes were consistent with the notion that the follicle on the left was the dominant follicle of a new wave, while the follicle on the right was the dominant follicle of a previous wave (modified with permission from Adams and Pierson, 1995).

overview of specific image analysis techniques and their applications in ovarian research and clinical diagnoses.

An ultrasonographic image is composed of thousands of picture elements, or pixels. Each pixel represents a discreet tissue reflector and can assume one of 256 shades of grey (ranging from black to white) in a 8-bit grey-scale image. The human eye can perceive "smoothness" of an image with increasing shades of grey, but can only distinguish between 18 and 20 shades of grey (Baxes, 1994). It is our contention that quantitative assessment of image data, through the use of high-resolution digital image acquisition and novel computer algorithms will allow researchers and clinicians to assess the physiology underlying ovarian follicular growth and development (Fig. 3).

The ultrasonographic appearance or image pattern of a tissue is termed echotexture and is determined by the histologic structure of the tissue (Powis and Powis, 1984; Ginther, 1995a; Singh et al., 1997, 1998; Singh and Adams, 2000). Computer algorithms have been designed specifically for analysis of ultrasound images in an effort to overcome the inconsistencies of subjective visual evaluation, and to provide a quantitative approach to grey-scale pixel-value analysis (Synergyne 2©, Version 1.1, WHIRL, Saskatoon, SK, Canada). These algorithms have been used extensively in studies characterizing the echotexture dynamics of ovarian structures at different phases of the follicular wave (Pierson and Adams, 1995; Singh et al., 1997, 1998; Tom et al., 1998a,b; Vassena et al., 2003).

To perform image analyses for the assessment of ovarian follicular function, each data point must be original, standardized, and easily incorporated into computer analysis software. The single most important issue in quantitative echotexture analysis is the standardization of the image during digital acquisition. Meaningful results have been obtained in vitro and in vivo in studies involving consistency in equipment (scanner and transducer)

and machine settings (near-field, far-field, and overall gain) for multiple examinations of the same structures over many days (Singh et al., 1997, 1998; Tom et al., 1998a,b). It is imperative that automatic post-processing image manipulations (e.g. frame correlation) are turned off during digital acquisition. Post-acquisition image normalization of pixel values involves re-scaling the pixel values of each image to span the entire scale of 0 (black) to 255 (white), and is done to correct for inconsistencies in image digitization spanning many days (Singh et al., 1998). However, the image normalization procedure cannot correct for inconsistencies in scanner settings. It is also critical that the computer graphics files are saved in a non-destructive and uncompressed format. Some file saving and file compression algorithms (for example, JPEG and GIF formats) approximate (i.e. lose) original data. Although such compression does not affect human perception of subtle tones of grey, it renders images unsuitable for echotexture analysis. Once the computer has digitally acquired an ultrasound image, it may be manipulated like any other graphics file. In our research institute, we use a series of digital processing steps that allows us to extract numerical data (spot and line analyses), enhance visual interpretation (region analysis and time-series analysis), and automate analysis of visual data (wavelet analysis and mathematical modeling).

3.1. Spot analysis

The simplest quantitative method to analyze the pixels comprising an ultrasound image is to select one or multiple small circular areas of interest (Fig. 4A). The computer can readily determine the precise values of all of the pixels (black = 0, white = 255) within the selected spot and provide an average value (mean pixel value) and a standard deviation value (pixel heterogeneity). This simple technique is conceptually similar to spot metering techniques in photography and may be used to compare the pixel values in various portions of the image or among images at different time points. For example, portions of the follicle antrum and wall from sequential images may be evaluated to detect changes characteristic of follicular dominance, growth, atresia, impending ovulation, or luteal development and regression (Pierson and Adams, 1995; Singh et al., 1997, 1998; Tom et al., 1998a,b; Vassena et al., 2003).

3.2. Line analysis

Similarly, a computer-generated line (thickness of 1 pixel, or more in some applications) may be drawn across a specified section of the image of the follicle to generate a graph of the grey-scale values of each pixel along the line. The graph depicts the amplitude of echoes located along the line (Fig. 4C). The two dimensions in this type of graphic display are distance (*X*-axis) and pixel value (PV) (*Y*-axis). Specific uses of this tool are to detect differences in a single follicle wall over time or among follicles. The antrum-wall interface of the follicle can be detected easily by identifying a sequential rise in grey-scale values along the line (Fig. 4C and D). Using the antrum-wall interface as a reference point, pixel values along the line can be divided into three segments, thus allowing separate analyses of the peripheral antrum, follicle wall, and stroma (Fig. 4C). Mean and peak pixel values, pixel heterogeneity, and area under the curve can be recorded separately for the three

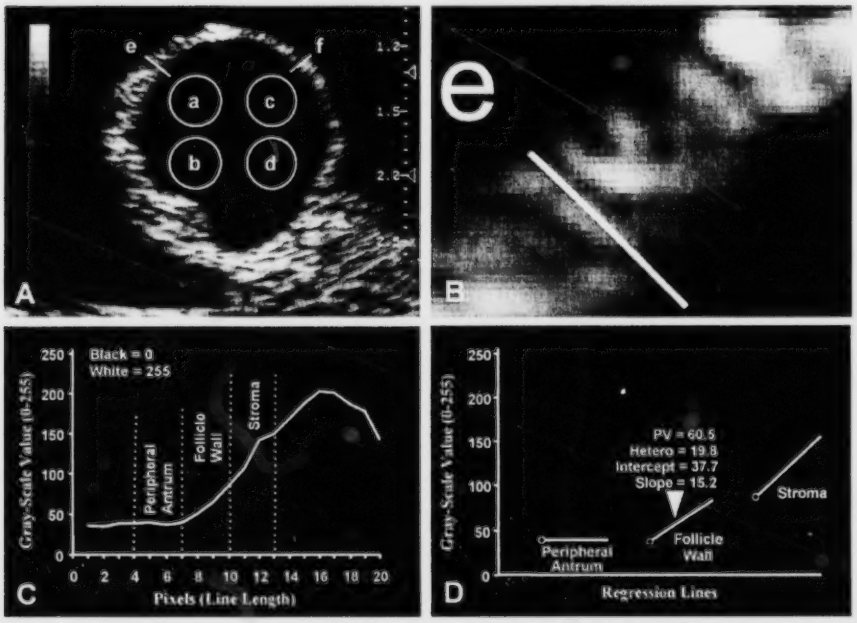

Fig. 4. (A, B) Computer-assisted analysis of ultrasound image of an ovarian follicle. Portion of the follicle wall from part A is enlarged in part B to display the picture-elements (pixels) forming the ultrasound image. Spot-analysis of the antrum is performed to measure the pixel value (black = 0, white = 255) and pixel heterogeneity by placing the measuring circle at four different locations (a, b, c, d) over the follicle antrum to cover approximately 20% of the area in each quadrant. (C) Line-analysis of the peripheral antrum, follicle wall and stroma was performed by placing a computer-generated line on the ultrasonographic image at 10 and 2 o'clock positions (e, f on part A, e on part B) and plotting the gray-scale values of pixels along the line. The pixel value graph was divided into three segments (peripheral antrum, follicle wall, stroma). The antrum-wall interface was used as a reference point. (D) Pixel value (PV), pixel heterogeneity (Hetero), and the intercept and slope of the regression line of each segment is recorded separately (modified with permission from Singh et al., 1998).

segments. In addition, pixel values along the line may be used to obtain intercept and slope values of a regression line for each of the three segments separately (Fig. 4D). To increase the precision and accuracy of analyses, numerical data may be averaged across multiple readings by placing the line-tool repeatedly across the wall of same follicle. It is important to note that care must be exercised to place the line in a region of the image free of artifacts (e.g. enhanced thorough transmission, shadowing, specular echoes, refraction, and beam-width artifacts; Kremkau, 1989; Ginther, 1995a; Heller and Jehle, 1995).

3.3. Region analysis

Region analysis of a follicle involves overlaying a pixel-by-pixel "mesh" onto a selected area (Fig. 5A) for recording pixel values at the points of intersection and generating a three-dimensional framework (Fig. 5B) or a wire-frame model. A computer generated "skin"

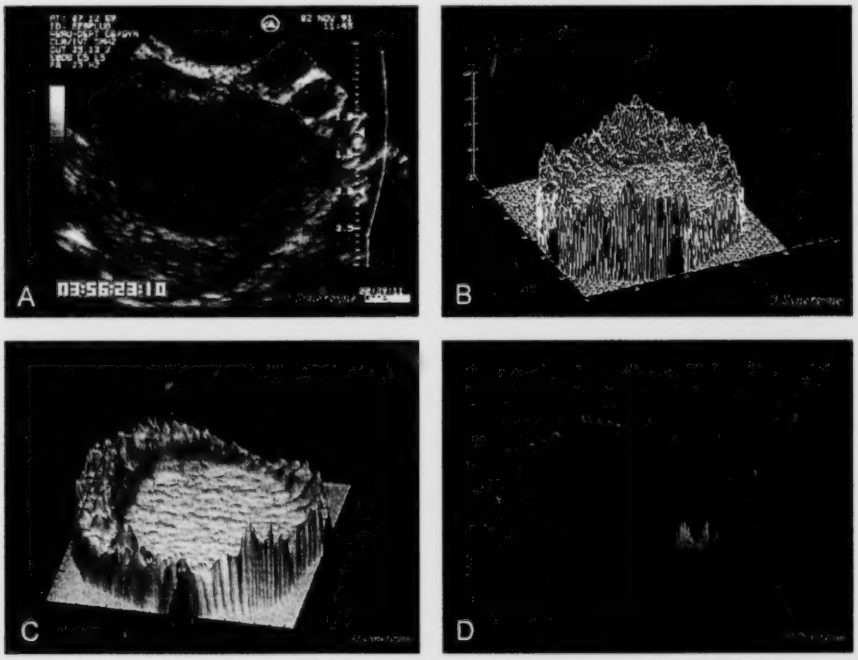

Fig. 5. Regional analysis of a single human preovulatory follicle. (A) Image of the follicle with follicle wall identified (black line). (B) Wire-frame model of the pixel by pixel mesh created from the image of the follicle. (C) Computer generated "skin" stretched over the wire-frame model. (D) Height shaded color algorithm added to the image to enhance visual appreciation and allow comparison of different zones with images of different follicles or images of the same follicle on different days.

can be placed over the mesh framework to yield a topographical surface (Fig. 5C). Shading algorithms may then be used to enhance the visual perception of surface features (Fig. 5C, surface shading, i.e. grey-scale or color assignment based on contour and direction) or echo values (height shading, i.e. grey-scale or color assignment based on "height" or brightness of pixels) of particular areas. Color application to the surfaces makes subtle differences in the surface contours easier to appreciate (Fig. 5D). In this type of display, the X and Y coordinates represent distance in two dimensions (i.e. length and width) and the Z-axis represents pixel value. The approach may develop into a diagnostic tool useful in clinical settings permitting instant visual assessment of ultrasonographic attributes associated with follicle health (viability or atresia).

3.4. Time series analysis

To provide information of image changes over time, a line is drawn across the follicle image, as previously described in the line analysis technique, to generate a two-dimensional graph (distance and pixel value) for the same follicle each day as it proceeds through the

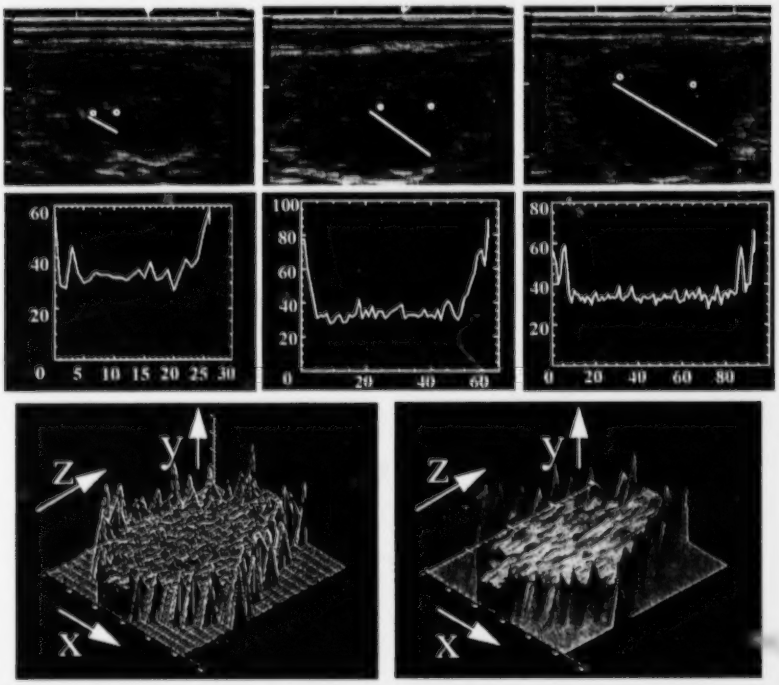

Fig. 6. Time-series analysis of a dominant anovulatory follicle. Individual follicles are identified (top three panels) on a daily basis and a graphic line representing their numerical values created (second three panels). The numerical values are concatenated and a wire-mesh framework created (lower left panel). The *X* dimension represents the diameter of the follicle including the follicle wall, the *Y* dimension represents the numerical pixel value, and the *Z* dimension represents the days of the cycle on which the follicle was uniquely identified. A surface rendering (skin) is placed over the framework to yield a conceptualization of the physiologic status of the follicle (lower right panel) from the time that the follicle was first identified, completes its growth phase, enters and passes through a static phase, and then regressed until it could no longer be identified. Addition of color shading algorithms adds to greatly enhanced visual perception.

phases of its growth or regression. Daily graphs are then concatenated or stung together to form a composite image in a third dimension (i.e. time). An example of this type of analysis is provided which illustrates a three-dimensional graph with follicle diameter along the *X*-axis, pixel value along the *Y*-axis and time along the *Z*-axis (Fig. 6). Shading algorithms may be applied to enhance contours, as described in region analysis. This type of analysis allows the assessment of echotextures of the follicular wall and antrum as they progress through physiologically important phases (e.g. day of dominant follicle selection, loss of dominance, atresia, day before ovulation). Time series analyses have been used in combination with other image analysis techniques to document marked differences in image characteristics associated with impending ovulation or atresia (Pierson and Adams, 1995) and detailed studies are ongoing.

3.5. Automated and semi-automated follicle recognition

A series of specialized algorithms for isolating images of follicles and follicle walls from surrounding ovarian tissues has been developed and implemented experimentally (Sarty et al., 1997; Sarty et al., 1998; Krivanek et al., 1998). This advance illustrates the potential for automated image analysis and data interpretation in ovarian biology. Study of ovarian imaging is particularly well-suited to automated analysis because of the magnitude of the interface between the follicular fluid and that of the soft tissues of the follicle wall. It is hoped that this approach may obviate the need for operator image interpretation and allow a standardized approach to interpreting ovarian and follicular physiology.

3.6. Wavelet packet texture analysis

Analysis of ultrasound images can be automated by wavelet packet texture recognition to allow clinicians to discriminate between healthy and atretic follicles. The technique involves convolve/decimate transformations (Misiti et al., 1996) of pixel values within the region of interest. In one study (Manivannan et al., 1999), the image of the follicular antrum was transformed into 85 wavelet images using three scale-levels. Wavelet classification of follicular status (healthy or atretic) appeared to be more accurate through the use of energy and standard deviation texture statistics than Euclidean minimum distance values. Although in the early stages of development, energy texture measurements based on three levels of wavelet packet transform, with or without further pure-scale transformations, hold potential as a diagnostic tool for classifying follicles.

3.7. Mathematical modeling

An interpretive mathematical approach may allow inference of hormone levels calculated from image data and thereby obviate the need for routine hormonal analyses. The procedure involves the integration of pixel-value image attributes into a comprehensive model which includes a mathematical description of the growth and regression of ovarian follicles in a competitive environment under the influence of FSH, LH, estradiol and other hormones (Sarty and Pierson, 1998, Fig. 7). In the model's simplest form, the growth of every follicle is governed by a first order, non-linear differential equation where the follicle maturity is measured by intrafollicular or circulating estradiol concentrations and image attributes (e.g. ultrasonography, MRI). Successful development of this type of modeling would provide the capability of assessing status on the basis of a single examination (diagnosis) and predicting the imminence of events (e.g. ovulation or the ovulatory potential of a follicle, optimal time for oocyte retrieval for IVF, optimal superstimulatory treatment regimen).

3.8. Physiological correlates of image attributes

In an initial characterization study, high-resolution ultrasound images of bovine follicles were obtained from ovaries excised at specific phases of follicular development (i.e. growth, early-static, late-static and regressing phases; Singh et al., 1998). The objective of the study was to determine whether quantitative changes in echotexture of dominant and subordinate

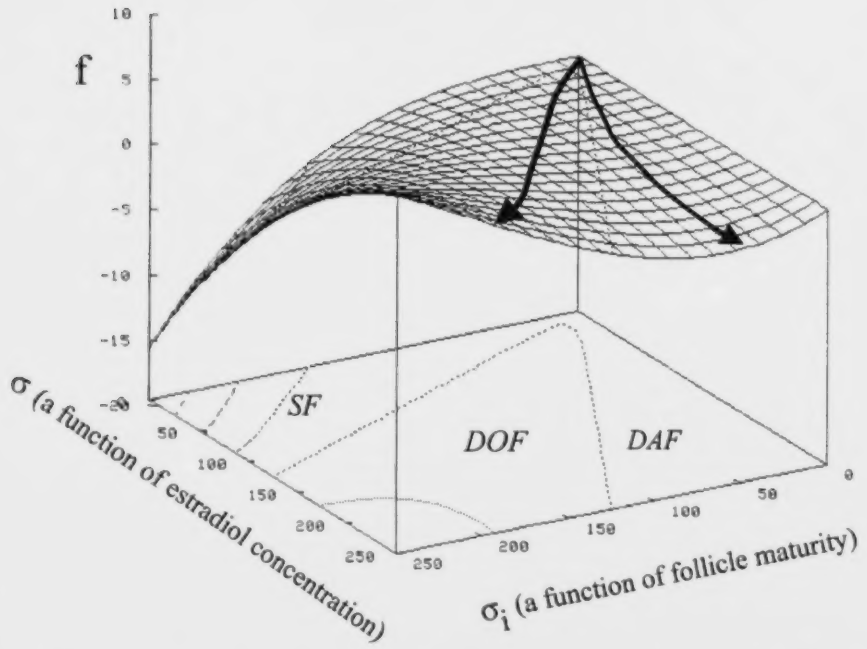

Fig. 7. Three dimensional parametric space model of ovarian follicular development. Maturity, m, of the follicles was equated to diameter. The rate of growth of the follicle z was determined by using central finite differences from the ultrasonographic time series data. The maturation rate (pg E_2 per hour calculated based on daily blood samples, ξ reflects circulating E_2 and ξ_i reflects maturity. SF represents the spatial area of subordinant follicles, DOF that occupied by dominant ovulatory follicles and DAF the area associated with dominant anovulatory follicles. The data were then plotted in the three dimensional (z, m, E_2) parametric space and interpolated to yield a mathematical maturation surface. We are interested in modifying this model to include image attributes, like echotexture and follicle size, and the effects of pathology and pharmacologic intervention which will yield interpretive models. On the surface framework, the line to the left illustrates the developmental history of a dominant ovulatory follicle while the line to the right illustrates the history of an dominant anovulatory follicle (modified from Sarty and Pierson, 1998).

follicles at different stages of development were reflective of their functional characteristics. Ultrasound images of the follicles were obtained under ideal conditions by placing the ovaries in a degassed water bath at standardized depth without interference from intervening tissues (i.e. rectal wall and adipose tissue). Follicular fluid was obtained after imaging the follicles for direct correlation. Estradiol concentrations in follicular fluid decreased from the growing to the late-static phase while progesterone content did not increase until follicles were in the final stages of regression. Detailed image analyses revealed that pixel values and heterogeneity of the antrum and wall were low in the growing, early-static and preovulatory phases of the dominant follicle. Conversely, pixel heterogeneity was characteristically high in late-static and regressing phases. The slope of the pixel value line spanning the follicle wall increased progressively from the growing to regressing phases (progressive

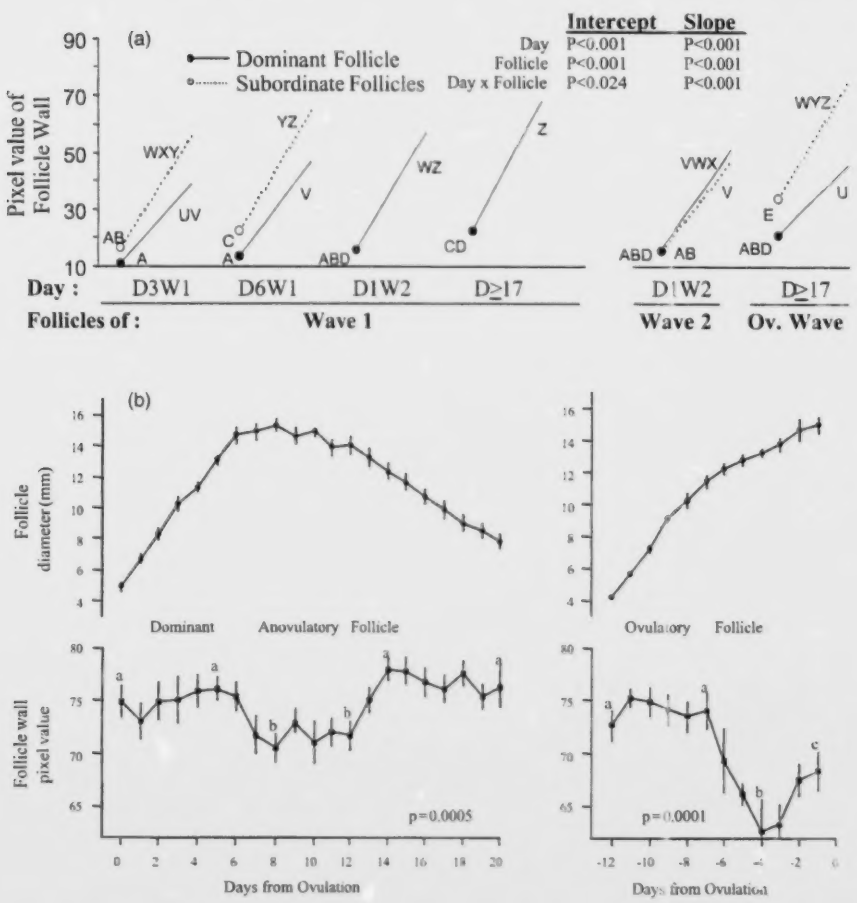

Fig. 8. (a) Intercept (circles) and slope of regression lines obtained by line-analysis of the follicle wall of the dominant and subordinate bovine follicles. Values with no common letters indicate significantly different values ($P < 0.05$) in intercept (A, B, C, D, E) and slope (U, V, W, X, Y, Z) (modified from Singh et al., 1998). (b) Diameter profiles and associated echotexture characteristics of dominant anovulatory and ovulatory follicles in cattle (mean ± S.E.M.; $n = 15$ observations per day). The growing (days 0–6) and regressing phases (more than day 12) of the dominant anovulatory follicle are separated by a static phase (days 6–12). Values with no common superscripts indicate significant difference ($P = 0.05$) (modified from Tom et al., 1998).

thinning of the follicle wall); the preovulatory dominant follicle had the thickest wall and the lowest slope (Fig. 8). Pixel heterogeneity of the antrum and wall, and the slope of the follicle wall regression line were negatively correlated ($P < 0.001$) to estradiol and the estradiol:progesterone ratio in follicular fluid. It was concluded that quantitative changes in the echotexture of ultrasound images occur concurrent with changes in functional and endocrine characteristics of the bovine follicles.

When the echotexture of bovine follicles was studied by in vivo imaging, time-related changes in the walls and antra of dominant ovarian follicles were temporally associated with developmental phases of the dominant follicle as determined by serial examinations (Tom et al., 1998b). The growing phase of the dominant follicle was characterized by high pixel values of the follicle wall and decreasing antral heterogeneity. The early static phase was marked by a drop in pixel values and increased antral heterogeneity during the late static phase. The regressing phase was characterized by high mean pixel values of the follicle wall and a heterogeneous antrum (Fig. 8b). The ovulatory follicle and the dominant anovulatory follicle of Wave 1 were similar in that pixel value of the wall began to decrease 6 days after wave emergence. However, pixel values of the wall were substantially lower in images of the preovulatory follicle than those observed in the anovulatory follicle, and values for the ovulatory follicle subsequently began to increase as ovulation approached. Perhaps the observed decreases in pixel values of the dominant follicle are indicative of ovulatory potential and presage the functional transition of the follicle to a corpus luteum. This hypothesis was supported by observations reported in other studies in which the walls of late growing anovulatory and ovulatory dominant bovine follicles (Adams and Pierson, 1995) and human preovulatory follicles (Martinuk et al., 1992) exhibited darker shades of grey than those of atretic follicles.

On the premise that changes in follicle image attributes (Singh et al., 1998; Tom et al., 1998; Manivannan et al., 1999) are direct reflections of changes in follicle structure, a histomorphometric study of bovine follicles of known stages of development was performed (Singh and Adams, 2000). The thickness of the follicle wall and granulosa layer, and vascularity of the theca interna of first wave dominant and subordinate follicles decreased progressively from growing and early-static phases to the regressing phase. In general, glandular cells, such as granulosa, theca and luteal cells appeared to be less echoic than the connective tissue stroma of the ovary. For instance, the ultrasound image of the mid-cycle bovine corpus luteum (maximal luteal cell volume at 56%) was darker than the early (angiogenic) or regressing luteal glands (luteal cells volume = 41%; Singh et al., 1997). Thus, thinning of the follicle wall during regression (Singh and Adams, 2000) resulted in a more rapid transition from the antrum (low pixel value) to the connective tissue stroma (high pixel value) leading to steeper slope values for the follicle wall (Fig. 8a). The preovulatory follicle had the thickest follicle wall, lowest cell densities in the granulosa and theca interna layers, and highest vascularity and edema in the theca interna (Singh and Adams, 2000), all of which are associated with the lowest pixel heterogeneity value and the lowest regression line slope value (Singh et al., 1998; Tom et al., 1998b). Follicle regression was characterized by detachment and release of granulosa cells from the follicle wall into the antrum (Singh and Adams, 2000). The antrum of healthy bovine follicles had very low mean pixel values while pixel heterogeneity was high in dominant follicles committed to atresia (Singh et al., 1998; Tom et al., 1998; Vassena et al., 2003) and in follicles that fail to ovulate in response to GnRH (Martinuk et al., 1992). Subordinate follicles have a brighter antrum with greater heterogeneity than their dominant counterparts (Singh et al., 1998; Vassena et al., 2003).

A relationship between image attributes and follicular function, and that between follicular status and oocyte competence have been documented (Salamone et al., 1999; Vassena, 2001). Hence, investigation of a relationship between image attributes of the ovarian follicle and the competence of the contained oocyte seemed a logical pursuit. Small to medium

antral follicles from bovine ovaries obtained at the slaughterhouse have been a primary source of oocytes for in vitro embryo production in cattle. However, such follicles are in different physiologic phases (i.e. growing, static or regressing) and presumably contain oocytes with varying degrees of developmental potential. In a recent study (Vassena et al., 2003), subordinate follicles whose oocytes produced 8- to 16-cell embryos had different echotextural characteristics than follicles whose oocytes did not produce embryos. A close relationship was found between the stage of development of subordinate follicles and their ultrasound echotexture. Together with the observation that oocyte competence is associated with follicular status, thus results provided important rationale for the use of ultrasound image analysis for identifying follicles that will produce competent oocytes. Although the sensitivity of this technique is not yet sufficient for use in a diagnostic setting, the identification of statistically significant endpoints has formed the basis to improve the imaging technique. Further, the use of the remote assessment of follicular status (Pierson and Adams, 1999) brings the prospects of providing useful information in near real-time well within the grasp of practitioners or research groups who may wish to have access to the biological information, but who do not wish to invest in the computer equipment and image analysis software required to make quantitative assessments of visual data.

4. Three-dimensional ultrasonography

Conventional B-mode ultrasonography involves continuous visualization of two-dimensional "slices" of a tissue (e.g. ovary, uterus, fetus) and mental reconstruction of the tissue structure in the third dimension. This is a technically demanding procedure that has impeded exploitation of the full potential of this type of medical imaging. Advances in computing power and transducer design have made three-dimensional (3D) imaging feasible. The first generation of commercially available 3D scanners is now available from at least five ultrasound imaging equipment manufacturers. In addition, an add-on computer that can be attached to the video output of a conventional ultrasound scanner to produce a 3D image system is available from one Canadian company.

In the simplest form of three-dimensional imaging, a standard linear-array or convex-array transducer head is moved mechanically over the tissue of interest and sequential images (e.g. 30 frames/s) are captured by computer digitization. The transducer head can be moved by free-hand rotation, or mechanically by an external stepper motor assembly, or by an enclosed stepper motor within the transducer head. The 2D images are then electronically stacked together to reconstruct the tissue volume. Specific algorithms may then be applied to enhance edge detection or isolate specific image features (e.g. make the fluid around a fetus transparent so that surface contours are visible) in near real-time (i.e. within seconds of image acquisition).

A tremendous advantage of 3D imaging is that once the images are acquired and reconstructed, the image may be rotated in all different planes and evaluated without continued scanning. Further, the 3D data sets can be used to provide volumetric measurements and permit acoustic sectioning or stripping of layers of image information, revealing both internal and external features (Fig. 9). Apart from computer-related issues, many technical challenges relative to anatomy (e.g. transducer size and configuration for transrectal or

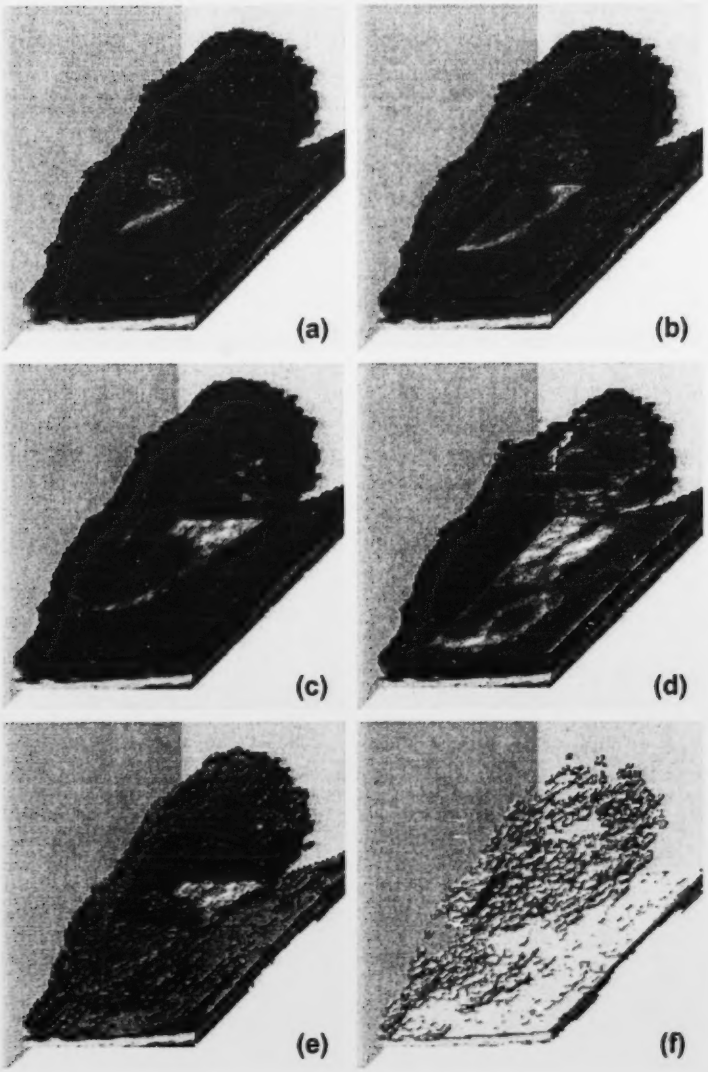

Fig. 9. Reconstructed 3D ultrasonographic images of a bovine ovary with cutout portions (a–d) to expose the topographic locations of ovarian follicles and corpus luteum. Different transparency levels (d–f) allow the 'dissolution' of various structures.

transvaginal use) and subject (e.g. animal movement during image acquisition) must be resolved before 3D ultrasonography becomes a practical in animal research applications.

Three basic approaches of image acquisition for 3D reconstruction have been used (Pierson, 1999).

4.1. Angular method

A standard linear-array or convex-array transducer head is moved at a constant speed through an arc of 45 –90°, and 2D images are acquired at regular intervals. A stepper motor housing that can be adapted to fit different commercial transducers has been designed for this type of imaging (Elango et al., 1997). The complete assembly remains stationary within the rectum or vagina while the transducer head moves for 0.5 to 6 s within the assembly. The original data set takes the form of a section of a cylinder (i.e. cylindrical coordinate system); therefore, biquadratic interpolation was used to transform the data set into one representing the shape of a cube (i.e. Cartesian coordinate system). Based on this design, commercial transducers have become available as completely enclosed dedicated probes; the design is conceptually analogous to a mechanical sector probe that contains an array of crystals rather than a single crystal.

4.2. Radial-rotation method

The radial method is suitable for use with end-fire convex-array transducers for transvaginal imaging, and involves movement of the transducer through one-half of a complete axial rotation (180° about its longitudinal axis (Kohler et al., 1997; Pierson, 1999). The reconstructed volume resembles the shape of a cone.

4.3. Matrix method

Angular and radial methods are limited by mechanical rotations of a row of crystals, but a matrix method makes use of electronic steering of ultrasound beams generated from a two dimensional matrix of crystals. Matrix probes are not yet available, but the "footprint" of such a probe will be large since the source is a sheet of piezoelectric crystals rather than a row. Matrix transducers may therefore be unsuitable for transrectal or transvaginal applications unless miniaturized versions can be developed. However, external approaches (e.g. transabdominal scanning) will be able to make use of faster acquisition times due to electronic steering of the beam.

5. Vascular imaging

The Doppler effect results from relative compression or rarefaction of sound waves from an approaching or departing source, respectively, and is the underlying reason for a change in acoustic pitch (frequency) from a moving sound source. The same principle is employed in ultrasonography to detect changes in the frequency of ultrasound waves reflected from clusters of blood cells flowing toward or away from the transducer face. Different

modalities of Doppler imaging allow detection of changes in blood velocity and direction of flow over time. Other indices used to describe vascular flow characteristics, such as resistance index, pulsatility index and systolic to diastolic ratios, may be calculated. Alterations in the vasculature of the ovary occur as large follicles and corpora lutea grow and regress, and microvascular changes occur in the tissues comprising follicle walls and the luteal glands (Singh et al., 1997; Singh and Adams, 2000). Hence, in addition to study of the vascular anatomy of ovarian follicles and corpus luteum (Pierson, 1992) by color-flow Doppler imaging, important physiologic processes may also be studied by characterizing changes in vascular dynamics over time (e.g. velocity, resistance, volume). Using color flow Doppler, a pronounced "ring" of vascularity has been detected around the developing human corpus luteum (Bächström et al., 1994). Vascularity became more prominent but resistance to vascular flow decreased as the corpus luteum matured. Conversely, color mapping patterns became much less pronounced and resistance increased during luteal regression. Three variations of Doppler imaging have been used to study vascular flow: Spectral Doppler is used to display vascular flow over time in a waveform; color-flow Doppler allows superimposition of directional vascular flow information on the B-mode grey-scale imaging; and power-flow Doppler is used to superimpose low velocity vascular flow information (without directional information) on the B-mode grey-scale imaging. Color-flow and power-flow Doppler images of ovarian structures are shown, illustrating the different types of information generated (Fig. 10). Power-flow Doppler has now been combined with color-flow Doppler to provide directional information for low velocity flow. We anticipate that as the techniques become more accessible in portable equipment, examination of blood flow patterns will become as routine and important in ovarian imaging as gray-scale ultrasonography is currently.

5.1. Spectral-flow Doppler ultrasonography

The spectral Doppler technique may involve a pulsed wave or a continuous wave. The advantage of the pulsed-wave spectral Doppler technique is that blood flow characteristics inside a specified vessel or at different regions within a cavity (e.g. heart chamber) can be measured precisely. This is made possible by the ability to place the Doppler gate (marker) at the desired anatomical location (e.g. within the lumen of vessels) using real-time ultrasonography. The ultrasound scanner then displays the spectral wave-forms over time in a different window. Quantitative data reflecting the characteristics of resistance, velocity and other characteristics may be generated. Research is just beginning on the assessment of echotexture characteristics and vascular patterns of individual preovulatory follicles (Pierson, 2000). Although data have not yet been critically evaluated, it appears that there was a gradual decrease in impedance to blood flow in the vessels immediately surrounding the human preovulatory follicle as ovulation nears. Immediately prior to ovulation, the perifollicular vessels were easily identified; however, it is necessary to visualize the perifollicular vessels in multiple planes due to their tortuous path (Collins et al., 1991; Pierson, 2001, in press).

5.2. Color-flow Doppler ultrasonography

Color Doppler scanners display local blood flow by assigning color to mean frequency shifts. This information is then superimposed on the real-time B-mode ultrasound image.

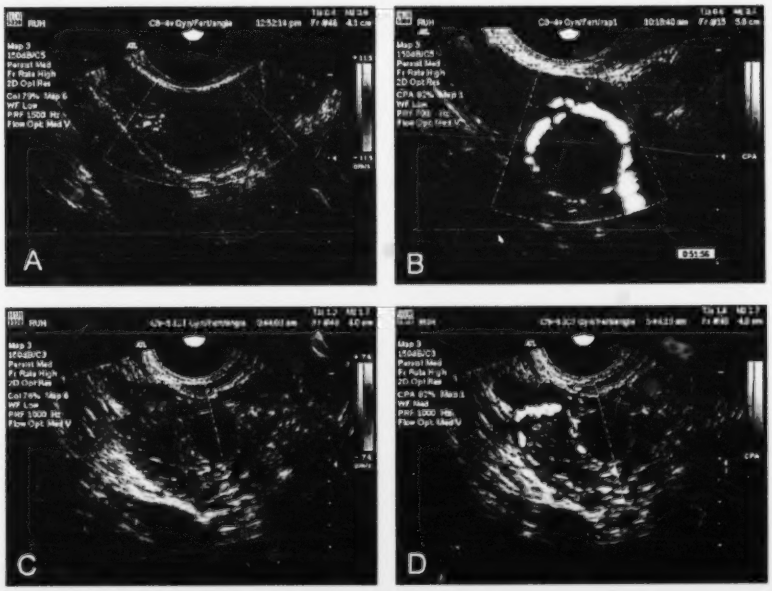

Fig. 10. Color flow (A, C) and power flow Doppler ultrasound images of imminently preovulatory follicles (A, B) demonstrating directional peri-follicular flow and recent sites of ovulation (C, D) demonstrating the vascular patterns around walls of the recently collapsed follicle/developing corpus luteum. In color flow images, blood flow toward the transducer is displayed in red, while flow away from the transducer is displayed in blue. In power flow images, all flow is displayed in a graded variant of yellow to orange colors.

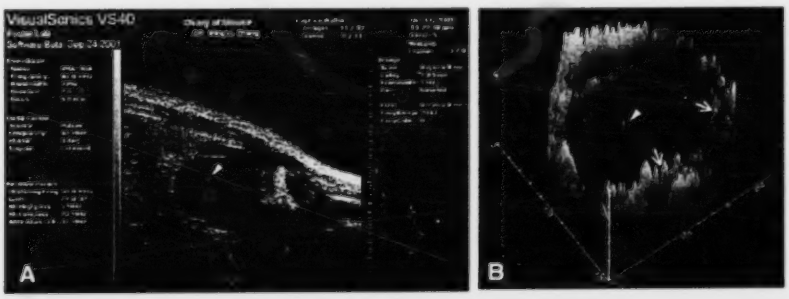

Fig. 11. A. High resolution image of an ovarian follicle of an anesthetized mouse obtained by transabdominal scanning with the ultrasound biomicroscope. The resolution obtained is far greater than that obtained in Fig. 3A. Compared to the image of bovine follicle (Fig. 3A), the mouse follicle was much smaller (2.4 mm vs. 15 mm) and was imaged in situ (live) rather than in vitro (excised ovary in a water bath). The cumulus-oocyte-complex (arrowhead) is visible in the center of the follicle. (B) Region analysis of the ultrasound image in A was performed and never-before seen details of the follicle wall (arrows) and the cumulus–oocyte-complex (arrowhead) are clearly evident.

Color-flow Doppler information is directional—different colors are displayed depending on whether the blood flow is towards or away from the transducer. Changes in blood flow around preovulatory follicles and during ovulation have been demonstrated by color flow Doppler ultrasonography in women (Brannstrom et al., 1998; Pierson, 2000; Pierson, unpublished observations; Fig. 10A and C). Evaluation of ovarian blood flow during follicular stimulation treatment in women (Balakier and Stronell, 1994; Nargund et al., 1996; Oyesanya et al., 1996; Van Blerkom et al., 1997) may be interpreted to mean that that peak velocity and resistive indices decrease as the diameter of the follicles increases. A strong correlation has been observed between oocyte recovery rates and the level of follicular vascularity, and it has been proposed that perifollicular blood flow may be used to choose the optimal time for hCG administration (Oyesanya et al., 1996). Likewise, color flow ultrasonography evaluation can also be used to detect physiological or pathological changes in the corpus luteum. Low rates of luteal blood flow were observed in non-pregnant women, while corpora lutea of pregnant women had the highest rates of blood flow (Zalud and Kurjak, 1990; Glock et al., 1995). One of the problems with color-flow imaging is that artifacts resulting from random noise during ultrasonography may resemble aberrant flow in any direction, thus obscuring true flow characteristics. Other artifacts stem from intestinal motion, turbulent flow and angle-dependent measurement errors.

5.3. Power-flow Doppler ultrasonography

A new application of the Doppler technique encodes the power in the Doppler in color. This is fundamentally different from the mean frequency shift employed by color-flow Doppler imaging. Since noise has uniformly low power, when power is written in color the noise appears uniform, not random. Hence, a uniformly colored background is imaged instead of a random distribution of color, therefore, the sensitivity in measuring vascular flow is improved (Zagzebski, 1996). Power-flow Doppler information is omni-directional—all flow in tissue is shown in a single color (Fig. 10B and D). Another advantage of power Doppler imaging is that it does not alias and is relatively angle independent. The use of power Doppler imaging of the reproductive organs is in its early stages. Preliminary studies on luteal function have been performed in normal women as well as women with luteal phase defects and luteinized unruptured follicle syndrome (Kupesic and Kurjak, 1997; Kupesic et al., 1997; Zaidi et al., 1995). Follicles which fail to ovulate (luteinized unruptured follicles) showed reduced blood flow in the follicle walls after the LH surge (Zaidi et al., 1995).

6. Ultrasound biomicroscopy

The combination of B-mode ultrasonography and computer-assisted echotexture analysis has the characteristics of an ideal diagnostic tool because it would permit continuous real-time evaluation of function/dysfunction. The current generation of commercial ultrasound machines provides a lateral resolution of 0.7–1.0 mm. This level of resolution is sufficient for many clinical uses, but the full potential of image analysis can be exploited with an instrument that provides microscopic resolution (i.e. <0.2 mm). A new

Canadian company has produced an ultrahigh-frequency ultrasound instrument capable of microscopic resolution (http://www.visualsonics.com). This "ultrasound biomicroscope" uses a single crystal to emit a sound wave with a frequency of 30–70 MHz and will produce an image with a resolution of 30–50 μm. To illustrate the potential of this emerging technology, a 0.7 mm ovarian follicle of an anesthetized mouse was imaged to reveal the structure of the cumulus-oocyte complex in vivo (Fig. 11). In addition, the instrument has spectral Doppler capability to measure blood flow through vessels as small as 50 μm in diameter. Major limitations of the biomicroscope are depth of penetration (approximately 10 mm at 30 MHz, 5 mm at 50 MHz), field of view (imaging width, 1 cm), and frame rate (8 frames/s). Close proximity of ovaries to the rectum and vagina in large domestic animals and humans will permit imaging with this instrument provided the instrument is used in conjunction with a conventional ultrasound machine for targeting the structures of interest. Echotexture analysis of images obtained with the ultrasound biomicroscope will, for the first time, allow the study of dynamic changes in the ovary at near histologic resolution. Serial imaging of microscopic structures over time will make accessible the study of mechanisms underlying biological events that have eluded reproductive biologists since the time of de Graaf (e.g. oocyte competence, early embryonic development and death, tubal fixation of the conceptus in humans, early luteal insufficiency, endometrial defects).

7. Magnetic resonance imaging (MRI)

7.1. Two-dimensional MRI

MRI has revolutionized the diagnosis of disease in many areas of human and veterinary medicine. MR images are less noisy and generally provide better resolution than ultrasound images (Sarty et al., 1996). Unlike speckle noise in ultrasound images (Abbott and Thurstone, 1979), the noise in MRI is not spatially correlated which makes it possible to improve signal-to-noise ratio by averaging. Magnetic resonance imaging is based upon relaxation of hydrogen protons in a large magnetic field after a radio-frequency pulse (RF) has deflected the proton spin transversely. During relaxation, T_1 recovery and T_2 decay occur simultaneously, but independently. T_1 recovery, also is known as spin-lattice relaxation, and means loss of RF pulse energy to the surrounding environment. T_2 decay, or spin–spin relaxation is the loss of energy to other protons or spins. Contrast in MR images is determined by T_1 and T_2 relaxation rates, and the proton density (PD) of tissue. The T_1 relaxation rate is the time it takes for 63% of the magnetization to recover to the longitudinal direction, or relax. The T_2 relaxation rate is the time for 63% of the transverse magnetization to be lost, or decay, while proton density is defined as the number of signal producing protons per unit volume of tissue. MR images can be weighted to preferentially reflect T_1 or T_2 relaxation as well as proton density. Two pulse sequence parameters, called T_R and T_E, govern the weighting in the image. T_1 recovery, T_2 decay and PD contribute to contrast in all MR images regardless of their weighting. An image weighted by T_1, T_2 or PD is produced by minimizing the effects of the other two processes.

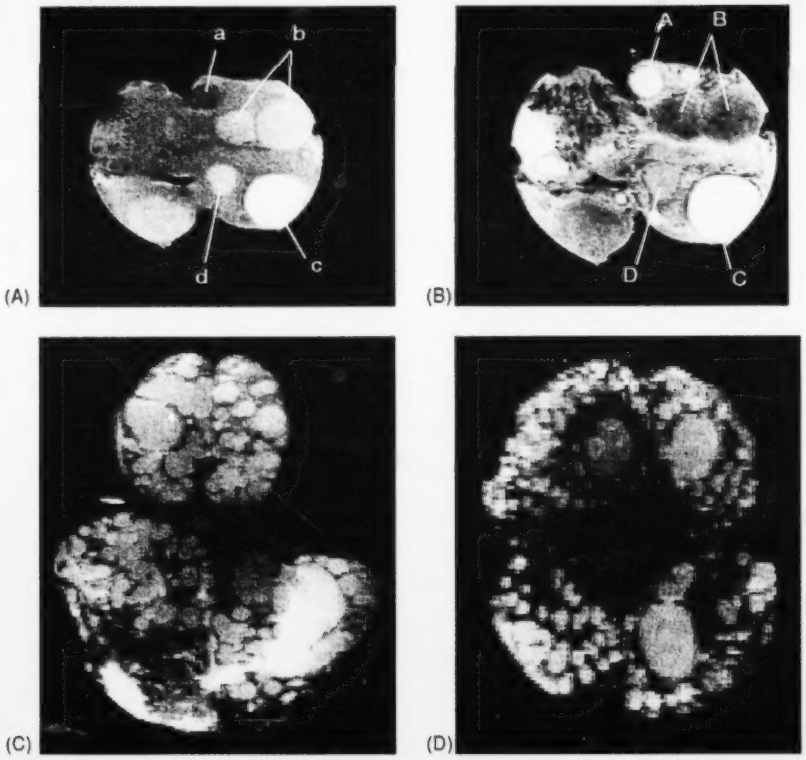

Fig. 12. MRI images of an ex vivo bovine ovary in two A, B and three C, D dimensions. Two-dimensional T_1-(A) and T_2-weighted images demonstrating follicles in different stages of atresia (a, A, b, B), the physiologically dominant follicle (c, C) and a corpus albicans (d, D). Small follicle of 2 mm diameter were easily visualized. Three dimensional images created using FISP and PFISP imaging. Follicles and luteal structures are clearly identifiable in all views. The images may be rotated 360° in the computer. Adapted from Hilton et al., 2000 and Sarty et al., 2000.

Reports on the use of MRI for studying the ovaries of domestic animals are virtually non-existent because of the cost of the equipment, the relatively small size of the ovaries compared to surrounding tissues, and the narrow tube diameter that prevents placement of large animals inside the magnetic coils. However, early prototypes of magnetic coils designed for intravaginal or intrarectal use are currently being developed and may make MRI as user-friendly as ultrasonography (Sarty et al., 2001, 2002). In an effort to explore the potential advantages of MRI imaging, bovine ovaries were surgically removed at defined times of follicular wave development (Sarty et al., 1996; Hilton et al., 2000, 2001). Two dimensional T_1 and T_2 weighted images are shown (Fig. 12A and B). Antral fluid of follicles imaged prior to dominant follicle selection and during atresia had long T_1 and T_2 relaxation times. As the time of selection of the dominant follicle approached, both the T_1 and T_2

times decreased. The T_1 time decreased in dominant follicles prior to ovulation. It appears that the T_1 value is inversely related to estradiol concentrations in the follicular fluid. Thus, conventional MRI relaxometry may be useful for identifying the physiologic status of ovarian follicles. We expect that improvements in MRI speed and resolution, combined with the use of specific purpose coils, will soon allow relaxometric observations of ovarian follicles in vivo.

7.2. *Three-dimensional MRI*

We have used three-dimensional MR imaging coupled with maximum intensity projection display to study the topographic distribution of follicles in bovine ovaries in vitro (Sarty et al., 2000, Fig. 12C and D). Ovaries were studied using fast imaging with steady state precession imaging sequences using maximum intensity projection which displayed three dimensional images as a cine-loop rotating in space. Presently, only the in vitro model system is functional, however, future developments making MRI fast and user-friendly make this a particularly exciting avenue of inquiry for the study of normal and pathologic ovarian function.

8. Diffraction-enhanced soft tissue X-ray imaging

The use of synchrotron light in medical imaging is an emerging field and has not been used previously to image ovarian tissue of any species. Excised bovine ovaries, and an

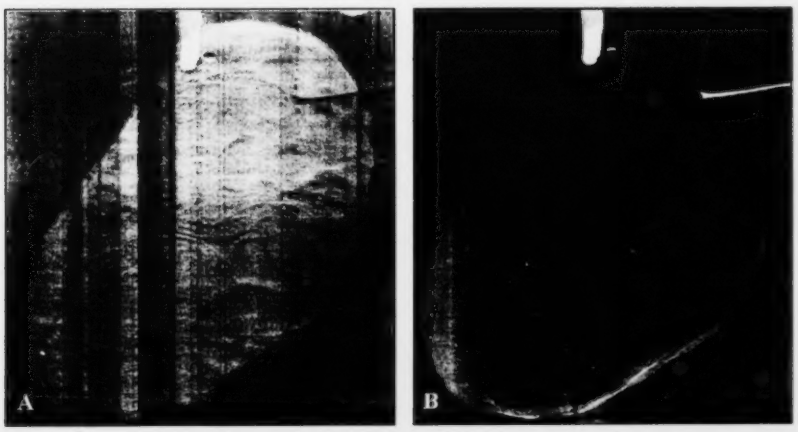

Fig. 13. Diffraction enhanced images (DEI) of a bovine ovary produced with X-rays on beamline ×15A of the National Synchrotron Light Source, Brookhaven National Laboratory, Upton, NY. Three antral follicles approximately 4 mm in diameter are indicated on the left, and a corpus luteum is outlined on the right. The images were acquired on opposite sides of the diffraction rocking curve, resulting in opposing contrast. Artifact was produced by a paper clip (at top) used to suspend the ovary in a plexiglass water bath (vertical striations).

early bovine embryo (about day 30 of gestation), were imaged using diffraction enhanced imaging (DEI) at 40 keV using a hard X-ray beamline at the National Synchrotron Light Source, Brookhaven National Laboratory, Upton, NY (Christensen et al., 2002). DEI is a new modality made possible by 2 unique characteristics of synchrotron light; i.e. the light is monochromatic (single wavelength) and polarized (single plane). In addition, synchrotron light is tremendously bright (in the order of 10^6 times brighter than the sun, at the surface of the sun) and therefore permits penetration and resolution not achievable with conventional radiography (e.g. angstroms to microns). DEI is particularly promising for soft tissue imaging because it provides exquisite contrast by using the X-ray bending properties of tissue, rather than the absorption properties. Follicles of various sizes and corpora lutea were apparent within the DE images of the ovaries; however, images of the embryo were unsatisfactory (Fig. 13). Future studies will utilize lower energies in an attempt to enhance feature contrast in ovarian and embryonic tissue, and employ computer-assisted image analysis to quantify texture differences within ovarian structures throughout the estrous cycle and pregnancy. At present, in vivo imaging of reproductive organs is not possible, but the ex vivo experiments have provided proof-of-principle that DEI of soft tissues is feasible. Future plans at the newly constructed Canadian Light Source include in vivo imaging of the ovaries in small and large animals using DEI and other modalities made possible by synchrotron light (e.g. phase contrast, K-edge subtraction).

9. Conclusion

Advancements in imaging technologies over the last two decades have ushered in a quiet revolution of research approaches to the study of ovarian structure and function. The most significant change in our understanding of the ovary have resulted from the use of ultrasonography which has enabled serial analyses in live animals. Our understanding of normal physiologic changes in the internal reproductive organs is now much clearer, as is our understanding of pathophysiologic changes. There has been rapid progression in the application of computer-assisted image analysis and mathematical modeling of the dynamic changes occuring within the ovary in vivo. In addition, new technologies, such as three-dimensional ultrasonography, two- and three-dimensional magnetic resonance imaging, ultrasound-based biomicroscopy and synchrotron-based techniques each have the potential to enhance our understanding almost to the cellular level.

Results of imaging studies over the past two decades have been promising and have far-reaching implications, well beyond the field of reproductive biology. We have developed convincing evidence to support the underlying hypothesis that echotexture characteristics of ovarian images reflect the endocrine and physiologic status of follicles, corpora lutea, and oocytes. Data collected by various modalities provide rationale for the notion that follicles have individual characteristics; i.e. at any given stage the structural and functional status of an individual follicle may be entirely different than that of others within its cohort. In addition, early results from studies designed to correlate echotexture analysis of the follicle wall with the developmental potential of the contained oocyte have been interpreted to mean that such differences may be useful for assessing or choosing oocytes for in vitro procedures. Ultimately, through the use of imaging, we would like to be able to unravel the mysteries of

ovarian function and dysfunction by detecting molecular and biochemical changes through changes in structure from the gross to cellular levels. Presently, image enhancement and echotexture analysis of images obtained by ultrasound biomicroscopy seems to be the most promising emerging technique that may allow in vivo examinations of early antral follicles and oocytes in near future. Perhaps one day we will be able to do the conceptual equivalent of Northern blot or in situ hybridization of follicular cells and gametes in vivo over time.

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References

Abbott, J.G., Thurstone, F.L., 1979. Acoustic speckle: theory and experimental analysis. Ultrason. Imaging 1, 303–324.

Adams, G.P., 1994. Control of ovarian follicular wave dynamics in cattle: implication for synchronization and superstimulation. Theriogenology 41, 25–30.

Adams, G.P., 1998. Control of ovarian follicular wave dynamics in mature and prepubertal cattle for synchronization and superstimulation. In: Proceedings of the Twentieth Congress of the World Association for Buiatrics. Sydney, Australia, vol. 2, pp. 595–605.

Adams, G.P., 1999. Comparative patterns of follicle development and selection in ruminants. Reproduction in domestic ruminants IV. J. Reprod. Fertil. Suppl. 54,17–32.

Adams, G.P., 2000. Developments in the use of ultrasonography in buiatrics. In: Proceedings of the Congresso Nazionale della Societa Italiana di Buiatria, vol. XXXII, 5–7 May 2000. Stresa, Italy, pp. 435–450.

Adams, G.P., Pierson, R.A., 1995. Bovine model for the study of ovarian follicular dynamics in humans. Theriogenology 43, 113–121.

Adams, G.P., Sumar, J., Ginther, O.J., 1990. Effects of lactational status and reproductive status on ovarian follicular waves in llamas (*Lama glama*). J. Reprod. Fertil. 90, 535–545.

Adams, G.P., Sumar, J., Ginther, O.J., 1991a. Form and function of the corpus luteum in llamas. Anim. Reprod. Sci. 24, 127–138.

Adams, G.P., Sumar, J., Ginther, O.J., 1991b. Hemorrhagic ovarian follicles in llamas. Theriogenology 35, 557–568.

Adams, G.P., Matteri, R.L., Ginther, O.J., 1992. Effect of progesterone on ovarian follicles, emergence of follicular waves and circulating follicle-stimulating hormone in heifers. J. Reprod. Fertil. 95, 627–640.

Adams, G.P., Evans, A.C.O., Rawlings, N.C., 1993. Follicular waves and circulating gonadotropins in 8-month old prepubertal heifers. J. Reprod. Fertil. 100, 27–33.

Atiomo, W.U., Pearson, S., Shaw, S., Prentice, A., Dubbins, P., 2000. Ultrasound criteria in the diagnosis of polycystic ovary syndrome (PCOS). Ultrasound Med. Biol. 26, 977–980.

Bächström, T., Nakata, M., Pierson, R.A., 1994. Ultrasonography of normal and abnormal luteogenesis. In: Jaffe, R., Pierson, R.A., Abramowicz, J.S. (Eds.), Imaging in Infertility and Reproductive Endocrinology. Lippincott, Philadelphia, pp. 143–154.

Baerwald, A.R., Adams, G.P., Pierson, R.A., 2002a. A new model for the menstrual cycle in women. In: Proceedings of the 11th World Congress on Human Reproduction, Montreal, Que. (abstract #29).

Baerwald, A.R., Adams, G.P., Pierson, R.A., 2002b. Folliculogenesis revisited: characteristics of ovarian follicular waves during the menstrual cycle. Fertil. Steril. 78(3) (Suppl. 1), S67.

Baerwald, A.R., Adams, G.P., Pierson, R.A., 2003. A new model for folliculogenesis during the menstrual cycle in women. Fertil. Steril., in press.

Balakier, H., Stronell, R.D., 1994. Color doppler assessment of folliculogenesis in vitro fertilization patients. Fertil. Steril. 62, 1211–1216.

Bartlewski, P.M., Beard, A.P., Cook, S.J., Rawlings, N.C., 1998. Ovarian follicular dynamics during anoestrus in ewes. J. Reprod. Fertil. 113, 275–285.

Baxes, G.A., 1994. Fundamentals of digital image processing. In: Digital Image Processing: Principles and Applications. Wiley, New York, pp. 13–36.

Bergfelt, D.R., Ginther, O.J., 1993. Relationship between FSH surges and follicular waves during the estrous cycle in mares. Theriogenology 39, 781–796.

Bergfelt, D.R., Brogliatti, G.M., Adams, G.P., 1998. Gamete recovery and follicular transfer (GRAFT) using transvaginal ultrasonography in cattle. Theriogenology 50, 15–25.

Bracher, V., Parlevliet, J., Fazelli, A.R., Pieterse, M.C., Vos, P.L., Dielmeman, S.J., Taverne, M.A., Colenbrander, B., 1993. Repeated transvaginal ultrasound-guided follicle aspiration in the mare. Equine Vet. J. Suppl. 15, 75–78.

Brannstrom, M., Zackrisson, U., Hagstrom, H.G., Josefsson, B., Hellberg, P., Granberg, S., Collins, W.P., Bourne, T., 1998. Preovulatory changes of blood flow in different regions of the human follicle. Fertil. Steril. 69, 435–442.

Brogliatti, G.M., Adams, G.P., 1996. Ultrasound-guided transvaginal oocyte collection in prepubertal calves. Theriogenology 45, 1163–1176.

Christensen, C.R., Singh, J., Adams, G.P., 2002. The use of diffraction enhanced imaging for bovine soft tissue. In: Proceedings of the Fifth Annual Users' Meeting and Workshops of the Canadian Light Source, 15–16 November 2002, University of Saskatchewan, Saskatoon, p. 26 (abstract #35).

Collins, W., Jurkovic, D., Bourne, T., Kurjak, A., Campbell, S., 1991. Ovarian morphology, endocrine function and intra-follicular blood flow during the peri-ovulatory period. Hum. Reprod. 6, 319–324.

Cook, N.L., Squires, E.L., Ray, B.S., Jasko, D.J., 1993. Transvaginal ultrasound-guided follicular aspiration of equine oocytes. Equine Vet. J. Suppl. 15, 71–74.

Elango, A., Gander, R.E., Adams, G.P., 1997. Design and development of a three-dimensional ultrasound transducer for veterinary applications. In: Proceedings of the Annual Conference of the Canadian Medical and Biological Engineering Society. Toronto, ON, Canada, pp. 74–75.

Evans, A.C.O., Adams, G.P., Rawlings, N.C., 1994a. Follicular and hormonal development in prepubertal heifers from 2 to 36 weeks of age. J. Reprod. Fertil. 102, 463–470.

Evans, A.C.O., Adams, G.P., Rawlings, N.C., 1994b. Endocrine and ovarian follicular changes leading up to the first ovulation in prepubertal heifers. J. Reprod. Fertil. 100, 187–194.

Fortune, J.E., 1993. Follicular dynamics during the bovine estrous cycle: a limiting factor in improvement of fertility? Anim. Reprod. Sci. 33, 111–125.

Gastal, E.L., Kot, K., Ginther, O.J., 1995. Ultrasound-guided intrafollicular treatment in mares. Theriogenology 44, 1027–1037.

Ginther, O.J., 1986. Ultrasonic imaging and reproductive events in the mare. Equiservices Publishing, Cross Plains, WI.

Ginther, O.J., 1992. Reproductive Biology of the Mare. 2nd ed. P224. Equiservices Publishing, Cross Plains, WI.

Ginther, O.J., 1995a. Ultrasonic Imaging and Animal Reproduction: Fundamentals Book 1. Equiservices Publishing, Cross Plains, WI, pp. 7–82, 35–40, 147–155.

Ginther, O.J., 1995b. Ultrasonic Imaging and Animal Reproduction: Horses. Equiservices Publishing, Cross Plains, WI, pp. 35–40, 77–83.

Ginther, O.J., Bergfelt, D.R., 1992. Ultrasonic characterization of follicular waves in mares without maintaining identity of individual follicles. J. Equine Vet. Sci. 12, 349–354.

Ginther, O.J., Kot, K., 1994. Follicular dynamics during the ovulatory season in goats. Theriogenology 42, 987–1001.

Ginther, O.J., Beg, M.A., Bergfelt, D.R., Donadeu, F.X., Kot, K., 2001. Follicle selection in monovular species. Biol. Reprod. 65, 638–647.

Ginther, O.J., Kastelic, J.P., Knopf, L., 1989a. Composition and characteristics of follicular waves during the bovine estrous cycle. Anim. Reprod. Sci. 20, 187–200.

Ginther, O.J., Knopf, L., Kastelic, J.P., 1989b. Temporal associations among ovarian events in cattle during oestrous cycles with 2 and 3 follicular waves. J. Reprod. Fertil. 87, 223–230.

Ginther, O.J., Knopf, L., Kastelic, J.P., 1989c. Ovarian follicular dynamics in heifers during early pregnancy. Biol. Reprod. 41, 247–254.

Ginther, O.J., Wiltbank, M.C., Fricke, P.M., Gibbons, J., Kot, K., 1996. Selection of the dominant follicle in cattle. Biol. Reprod. 55, 1187–1194.

Ginther, O.J., Kot, K., Kulick, L.J., Wiltbank, M.C., 1997. Sampling follicular fluid without altering follicular status in cattle: oestradiol concentrations early in a follicular wave. J. Reprod. Fertil. 109, 181–186.

Glock, J.L., Blackman, J.A., Dadger, G.J., Brumstead, J.R., 1995. Prognostic significance of morphologic changes of the corpus luteum by transvaginal ultrasound in early pregnancy monitoring. Obstet. Gynecol. 85, 37–41.

Heller, M, Jehle, D., 1995. Ultrasound in Emergency Medicine. Saunders, Philadelphia, pp. 1–40.

Hilton, J.E., Sarty, G.E., Adams, G.P., Pierson, R.A., 2000. Magnetic resonance image attributes of the bovine ovarian follicle antrum during development and regression. J. Reprod. Fertil. 120, 311–323.

Jocelyn, H.D., Setchell, B.P., 1972. Regnier de Graaf on the human reproductive organs. An annotated translation of Tractatus de Virorum Organis Generationi Inservientibus (1668) and De Mulierub Organis Generationi Inservientibus Tractatus Novus (1962). J. Reprod. Fertil. Suppl. 17, 1–222.

Hinrichs, K., DiGiorgio, L.M., 1991. Embryonic development after intrafollicular transfer of horse oocyte. J. Reprod. Fertil. Suppl. 44, 369–374.

Hoare, E.K., Parker, S.E., Flood, P.F., Adams, G.P., 1997. Ultrasonic imaging of reproductive events in muskoxen. Rangifer 17, 119–123.

Kastelic, J.P., Ginther, O.J., 1989. Fate of conceptus and corpus luteum after induced embryonic loss in heifers. J. Am. Vet. Med. Assoc. 194, 922–928.

Kastelic, J.P., Bergfelt, D.R., Ginther, O.J., 1990a. Relationship between ultrasonic assessment of the corpus luteum and plasma progesterone concentration in heifers. Theriogenology 33, 1269–1278.

Kastelic, J.P., Pierson, R.A., Ginther, O.J., 1990b. Ultrasonic morphology of corpora lutea and central cavities during the estrous cycle and early pregnancy in heifers. Theriogenology 34, 487–498.

Knopf, L., Kastelic, J.P., Schallenberger, E., Ginther, O.J., 1989. Ovarian follicular dynamics in heifers: test of two-wave hypothesis by ultrasonically monitoring individual follicles. Dom. Anim. Endocr. 6, 111–119.

Kohler, J., Gander, R.E., Pierson, R.A., 1997. Development of intravaginal three-dimensional ultrasonography. In: Proceedings of the Conference of the Canadian Medical and Biological Engineering Society, pp. 134–135.

Kot, K., Gibbons, J.R., Ginther, O.J., 1995. A technique for intrafollicular injection in cattle: effects of hCG. Theriogenology 44, 41–50.

Kremkau, F.W., 1989. Diagnostic Ultrasound: Principles, Instruments, and Exercises. Saunders, Philadelphia, pp. 1–50, 147–176, 219–231.

Krivanek, A., Liang, W., Sarty, G., Pierson, R.A., Sonka, M., 1998. Automated follicle analysis in ovarian ultrasound medical imaging 1998: image processing. In: Proceedings of the International Society for Optical Engineering (SPIE), vol. 3338, pp. 588–596.

Kupesic, S., Kurjak, A., Vujisic, S., Petrovic, Z., 1997. Luteal phase defect: comparison between Doppler velocimetry, histological and hormonal markers. Ultrasound Obstet. Gynecol. 9, 105–112.

Kupesic, S., Kurjak, A., 1997. The assessment of normal and abnormal luteal function by transvaginal color Doppler sonography. Eur. J. Obstet. Gynecol. Reprod. Biol. 72, 83–87.

Manivannan, U., Sarty, G.E., Sirounis, H., Singh, J., Adams, G.P., Pierson, R.P., 1999. Physiologic classification of bovine ovarian follicles with wavelet packet texture analysis. SPIE 3661, 1238–1245.

Martinuk, S.D., Chizen, D.R., Pierson, R.A., 1992. Ultrasonographic morphology of the human preovulatory follicle wall prior to ovulation. Clin. Anat. 5, 1–14.

McCorkell, R.B., MacDougall, L., Adams, G.P., 2001. Transrectal ultrasonography in female Wapiti (*Cervus elaphus*): a feasibility study. In: Renecker, L.A., Renecker, T.A. (Eds.), Game Conservation and Sustainability: Biodiversity, Management, Ecotourism, Traditional Medicine and Health. Stratford, ON, Canada, pp. 282–290.

McCorkell, R.B., MacDougall, L., Adams, G.P., 2002. Ovarian follicle development in Wapiti (*Cervus elaphus*) during the anestrous season. Theriogenology, in press.

Misiti, M., Misiti, Y., Oppenheim, G., Poggi, J.M., 1996. Wavelet Toolbox Users's Guide. The Math Works Inc., Natick, Mass.

Nargund, G., Bourne, T., Doyle, P., Parsons, J., Cheng, W., Campbell, S., Collins, W., 1996. Associations between ultrasound indices of follicular blood flow, oocyte recovery and preimplantation embryo quality. Hum. Reprod. 11, 109–113.

Oyesanya, O.A., Parsons, J.H., Collins, W.P., Campbell, S., 1996. Prediction of oocyte recovery rate by transvaginal ultrasonography and color Doppler imaging before human chorionic gonadotropin administration in vitro fertilization cycles. Fertil. Steril. 65, 806–809.

Pierson, R.A., 1992. From ovulation to implantation. In: Jaffe, R., Warsof, S. (Eds.), Color Doppler Imaging in Obstetrics and Gyanecology. McGraw-Hill, New York, pp. 35–60.

Pierson, R.A., 1999. Three-dimensional ultrasonography of the embryo and fetus. In: Jaffe, R., Bui, T.-H. (Eds.), Fetal and Neonatal Ultrasonography. Parthenon Publishing Group, New York, pp. 317–326.

Pierson, R.A., 2000. Imaging technology in assisted reproduction. In: Wolf, D., Zelinski-Wooten, M. (Eds.), Assisted Reproduction and Nuclear Transfer in Mammals. Humana Press, Totowa, NJ, pp. 95–122.

Pierson, R.A., Adams, G.P., 1995. Computer-assisted image analysis, diagnostic ultrasonography and ovulation induction: strange bedfellows. Theriogenology 43, 105–112.

Pierson, R.A., Adams, G.P., 1999. Remote assessment of ovarian response and follicular status using visual data analysis of ultrasonographic images. Theriogenology 51, 47–57.

Pierson, R.A., Chizen, D.R., 1994. Transvaginal ultrasonographic assessment of normal and aberrant ovulation. In: Jaffe, R., Pierson, R.A., Abramowicz, J.S. (Eds.), Imaging in Infertility and Reproductive Endocrinology. Lippincott, Philadelphia, pp. 129–142.

Pierson, R.A., Ginther, O.J., 1984. Ultrasonography of the bovine ovary. Theriogenology 21, 495–504.

Pierson, R.A., Ginther, O.J., 1985. Ultrasonic evaluation of the corpus luteum of the mare. Theriogenology 23, 795–806.

Pierson, R.A., Ginther, O.J., 1986. Ovarian follicular populations during early pregnancy in heifers. Theriogenology 26, 649–659.

Pierson, R.A., Ginther, O.J., 1987a. Follicular populations during the estrous cycle in heifers: Part I. Influence of day. Anim. Reprod. Sci. 124, 165–176.

Pierson, R.A., Ginther, O.J., 1987b. Follicular populations during the estrous cycle in heifers. Part II. Influence of right and left sides and intraovarian effect of the corpus luteum. Anim. Reprod. Sci. 14, 177–186.

Pierson, R.A., Ginther, O.J., 1988a. Follicular populations during the estrous cycle in heifers. Part III. Time of selection of ovulatory follicle. Anim. Reprod. Sci. 16, 81–95.

Pierson, R.A., Ginther, O.J., 1988b. Ultrasonic imaging of the ovaries and uterus in cattle. Theriogenology 29, 21–37.

Pieterse, M.C., Kappen, R.A., Kruip, A.M., Taverne, M.A.M., 1988. Aspiration of bovine oocyte during transvaginal ultrasound scanning of the ovaries. Theriogenology 30, 751–762.

Powis, R., Powis, W., 1984. A Thinker's Guide to Ultrasonic Imaging. Urban and Schwartzenberg Inc., Baltimore, MD.

Rajakoski, E., 1960. The ovarian follicular system in sexually mature heifers with special reference to seasonal, cyclical, and left-right variations. Acta Endocr. Copenh. Suppl. 52, 7–68.

Rajamahendran, R., Taylor, C., 1990. Characterization of ovarian activity in postpartum dairy cows using ultrasound imaging and progesterone profiles. Anim. Reprod. Sci. 22, 171–180.

Ravindra, J.P., Rawlings, N.C., Evans, A.C.O., Adams, G.P., 1994. Ultrasonographic study of ovarian follicular dynamics in ewes during the oestrous cycle. J. Reprod. Fertil. 101, 501–509.

Salamone, D.F., Adams, G.P., Mapletoft, R.J., 1999. Changes in the cumulus–oocyte complex of subordinate follicles relative to follicular wave status in cattle. Theriogenology 52, 549–561.

Sarty, G.E., Pierson, R.A., 1998. Analysis of ovarian follicular response to superstimulation in a three-dimensional parametric space. Fertil. Steril. 70 (Suppl 3), S183.

Sarty, G.E., Adams, G.P., Pierson, R.A., 2000. Three-dimensional magnetic resonance imaging for the study of ovarian function in bovine in vitro model. J. Reprod. Fertil. 119, 69–75.

Sarty, G.E., Baerwald, A.R., Loewy, J., Pierson, R.A., 2002. External versus internal magnetic resonance imaging methods for the study of ovarian structure and function. MAG*MA, in press.

Sarty, G.E , Hess, A.R., (Baerwald), Loewy, J., Pierson, R.A., 2001. External versus intravaginal mri for ovarian imaging. In: Proceedings of the 47th Annual Meeting of the Canadian Fertility and Andrology Society, Whistler, BC (Abstract 58a).

Sarty, G.E., Kendall, E.J., Pierson, R.A., 1996. Magnetic resonance imaging of bovine ovaries in vitro. Mag. Res. Mat. Physics Biol. Med. (MAG*MA) 4, 205–211.

Sarty, G.E., Liang, W., Sonka, M., Pierson, R.A., 1997. The Development of a Semi-Automated Segmentation of Ovarian Follicular Images Using a Knowledge-Based Algorithm. Medical Imaging 1997: Image Processing. In: Proceedings of the International Society for Optical Engineering (SPIE), 3034, pp. 822–829.

Sarty, G.E., Liang, W., Sonka, M., Pierson, R.A., 1998. Semi-automated segmentation of ovarian follicular ultrasound images using a knowledge-based algorithm. J. Ultrasound Med. Biol. 24, 27–42.

Savio, J.D., Boland, M.P., Hynes, N., Roche, J.F., 1990. Resumption of follicular activity in the early postpartum period of dairy cows. J. Reprod. Fertil. 88, 569–579.

Savio, J.D., Keenan, L., Boland, M.P., Roche, J.F., 1988. Pattern of growth of dominant follicles during the estrous cycle of heifers. J. Reprod. Fertil. 83, 663–671.

Singh, J., Adams, G.P., 2000. Histomorphometry of dominant and subordinate bovine ovarian follicles. Anat. Rec. 258, 58–70.

Singh, J., Pierson, R.A., Adams, G.P., 1997. Ultrasound image attributes of the bovine corpus luteum: structural and functional correlates. J. Reprod. Fertil. 109, 35–44.

Singh, J., Pierson, R.A., Adams, G.P., 1998. Ultrasound image attributes of bovine ovarian follicles: endocrine and functional correlates. J. Reprod. Fertil. 112, 19–29.

Sirois, J., Fortune, J.E., 1988. Ovarian follicular dynamics during the estrous cycle in heifers monitored by real-time ultrasonography. Biol. Reprod. 39, 308–317.

Spicer, L.J., Echternkamp, S.E., 1986. Ovarian follicular growth, function and turnover in cattle: a review. J. Anim. Sci. 62, 428–451.

Stock, A.E., Fortune, J.E., 1993. Ovarian follicular dominance in cattle: relationship between prolonged growth of the ovulatory follicle and endocrine parameters. Endocrinology 132, 1108–1114.

Tom, J.W., Pierson, R.A., Adams, G.P., 1998a. Quantitative echotexture analysis of bovine corpora lutea. Theriogenology 49, 1345–1352.

Tom, J.W., Pierson, R.A., Adams, G.P., 1998b. Quantitative echotexture analysis of bovine ovarian follicles. Theriogenology 50, 339–346.

Van Blerkom, J., Antczak, M., Schrader, R., 1997. The developmental potential of the human oocyte is related to the dissolved oxygen content of follicular fluid: association with vascular endothelial growth factor levels and perifollicular blood flow characteristics. Hum. Reprod. 12, 1047–1055.

Vassena, R., 2001. Bovine Oocyte Competence. M.Sc. Thesis, University of Saskatchewan, Saskatoon, Canada.

Vassena, R., Adams, G.P., Mapletoft, R.J., Pierson, R.A., Singh, J., 2003. Ultrasound image characteristics of ovarian follicles in relation to oocyte competence and follicular status in cattle. Anim. Reprod. Sci. 76, 25–41.

Wikland, M., Nilsson, L., Hansson, R., Hamberger, L., Janson, P.O., 1983. Collection of human oocytes by the use of sonography. Fertility Sterility 38, 603–608.

Zagzebski, J., 1996. Doppler instrumentation. In: Essentials of Ultrasound Physics. Mosby-Year Book, Inc., St. Louis, MO, pp. 87–108.

Zaidi, J., Jurkovic, D., Campbell, S., Collins, W., McGregor, A., Tan, S.L., 1995. Luteinized unruptured follicle: morphology, endocrine function and blood flow changes during the menstrual cycle. Hum. Reprod. 10, 44–49.

Zalud, I., Kurjak, A., 1990. The assessment of luteal blood flow in pregnant and non-pregnant women by transvaginal color Doppler. J. Perinat. Med. 18, 215–221.

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Morphology and developmental competence of bovine oocytes relative to follicular status

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Abstract

In cattle, follicle dimension has been used as the main criterion for selection of oocytes for in vitro embryo production. However, follicles with similar diameters may be in very different physiologic phases. The aim of this study was to investigate whether morphology and developmental competence of cumulus–oocyte complexes (COCs) are related to the phase of development of the follicle, and presence of the corpus luteum (CL) or the dominant follicle in the ovary from which the COCs were collected. Cows ($n = 143$) were given a luteolytic dose of $PGF_{2\alpha}$ and 8 days later underwent transvaginal ultrasound guided ablation of follicles ≥4 mm to induce emergence of a new follicular wave. Cows ($n = 10$–20 per replicate) were slaughtered on Day 2, 3, 5 or 7 (Day 0 = follicular wave emergence), equivalent to the growing, early static, late static, and regressing phases of subordinate follicle development. COCs were collected from subordinate follicles ≥3 mm, were classified as denuded, degenerated or healthy, and underwent IVM–IVF–IVC. The proportion of oocytes that developed to the blastocyst stage was higher ($P < 0.05$) in those collected on Day 5 after wave emergence (23%) than on Day 2 (12%), 3 (13%) or 7 (16%). Data did not support the hypothesis of a local effect of the CL or dominant follicle. We conclude that a positive relationship exists between early follicular regression and oocyte competence. Moreover, morphologic characteristics of oocyte quality used in this study were not predictive in identifying competent oocytes.
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Keywords: Oocyte competence; Follicular wave; Follicles; In vitro fertilization; Embryo

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1. Introduction

For more than a decade, calves have been produced from oocytes after IVM, IVF, and IVC [1–3]. However, the proportion of oocytes that develop to the blastocyst stage in vitro has not improved over time [4]. Much attention has been given to the in vitro environment; however, the inability to improve in vitro embryo production may be a result of the inherent developmental competence of oocytes collected from small follicles (2–6 mm diameter; [5]). The majority of cumulus–oocyte complexes (COCs) collected from 2 to 6 mm follicles appear to mature in vitro, but many are not capable of producing blastocysts [6]. Oocyte competence, defined as the ability of an oocyte to develop to the blastocyst stage in vitro, has been examined in relation to follicular diameter in cattle [7,8], and current production methods use follicle diameter as the main criterion for selecting COC for in vitro embryo production. However, follicles of the same diameter can be in quite diverse physiological phases, depending on the stage of development in the follicular wave at the time of collection.

Emergence of a follicular wave is defined as the synchronous growth of a cohort of follicles [9–11]. Follicular growth during the estrous cycle in the cow is characterized by two or three follicular waves. During each wave, a cohort of follicles begins to grow beyond 4 mm in diameter. From this cohort, a single follicle becomes dominant and continues to grow while the remaining follicles in the cohort begin to regress. The hypothesized mechanism of selection of the dominant follicle involves inter-follicular competition for FSH, and the liberation of the incipient dominant follicle from FSH dependence through the acquisition of LH responsiveness [12]. After the wave inducing surge of FSH [13], competition among follicles becomes acute; the first follicle to acquire LH receptors becomes dominant while those depending on FSH regress in response to decreasing concentrations of circulating FSH. The competitive phase ends around Day 5 of the wave, after which the subordinate follicles are committed to regression [13,14]. Ablation of the dominant follicle by either cautery [13,14] or puncture [15,16] resulted in the emergence of a new follicular wave within the next 2 days (mean, 1.5 days after ablation). Ultrasound guided transvaginal follicular ablation was used in the present study to elicit new wave emergence.

The purpose of this study was to test the hypotheses that oocyte competence is related to the phase of the follicular wave in which it is collected rather than to the dimension of the follicle from which it is derived, that oocyte competence is influenced locally by the presence of the CL or the dominant follicle in the ovary from which it was collected, and that morphological characteristics of the COCs are related to the stage of the follicular wave and its developmental competence.

2. Materials and methods

2.1. Animals and grouping

Cross-bred beef cows (n = 152, Bos taurus), 4–14 years of age, were used during July, August, and September. The cows were kept in three outdoor paddocks on a single farm in southern Saskatchewan, and maintained on a rising plane of nutrition (barley and hay) in preparation for slaughter. After initial ultrasonographic examination of the reproductive

system (Aloka SSD-500 with a 5 MHz linear-array transducer), nine cows were excluded because of advanced pregnancy. Thirteen of the remaining 143 cows were ≤70 days pregnant and were given $PGF_{2\alpha}$ to induce abortion (25 mg dinoprost; Lutalyse; Pharmacia & Upjohn Animal Health, Orangeville, Ont., Canada). They were included in the last replicate of the study, at least 40 days after induced abortion. Cows were assigned randomly to groups in which ovaries were to be collected at slaughter on Day 2, 3, 5 or 7 of the follicular wave (Day 0 = wave emergence). Cows (10–20 per replicate) representing 2 day-groups, were synchronized to permit slaughter on a given day. Cows were thus distributed among eight replicates. Synchronization involved a luteolytic dose of $PGF_{2\alpha}$ followed 8 days later by transvaginal ultrasound guided ablation of all follicles ≥4 mm. The day after follicle ablation was considered to be the day of wave emergence [15]. The goal of the synchronization scheme was to electively induce a wave of follicular development during the luteal phase in all animals.

2.2. COC collection and classification

About 2 h before the cows were sent to the abattoir, the ovaries were examined by transrectal ultrasonography to record the dimensions and positions of the dominant and subordinate follicles and the corpus luteum (CL). Cows were grouped so that the ovaries could be collected in a time range not exceeding 30 min after slaughter. Each ovary was placed in an individual plastic bag filled with phosphate-buffered saline (PBS) + 1% (w/v) antibiotic/antimycotic (10,000 IU penicillin, 10 mg streptomycin and 25 μg amphotericin B/ml; Sigma–Aldrich Canada Ltd., Oakville, Ont., Canada). Each bag was marked with an identification number different from the ear tag number so that the investigator was blinded from day groupings. The ovaries were maintained at 30 °C and transferred to the laboratory within 3 h of collection. The COCs were aspirated into a 15 ml glass vial containing 3 ml of warm collection medium (TCM 199 + 0.75% kanamycin) using an 18 g 1.5 in. needle and a vacuum pump with a continuous controlled flow-rate of 35 ml/min. The needle was rinsed with warm collection medium before and after aspiration of each follicle to minimize sticking of the COC to the internal surfaces of the needle and tubing. The dominant follicle and, when in doubt, the largest subordinate follicle were aspirated separately. The COCs from all subordinate follicles ≥3 mm in the same ovary were collected and pooled in the same tube. The COCs were evaluated morphologically at a magnification 400× and categorized as denuded, degenerate or healthy. Denuded oocytes were defined as those with less than one complete surrounding layer of granulosa cells. Degenerate COCs were those with one or more of the following characteristics: very expanded cumulus, ≤2 surrounding layers of granulosa cells, pyknotic granulosa cell nuclei, fragmented oocyte nucleus, misshapen, partially absent or vacuolated oocyte cytoplasm, empty zona pellucida. Healthy COCs were those with ≥3 surrounding layers of granulosa cells, a compact cumulus, smoothly granular oocyte cytoplasm, and no irregularities of the oocyte nucleus.

2.3. In vitro maturation, fertilization and culture (IVM, IVF, IVC)

All COCs were matured, fertilized, and cultured, regardless of initial morphology. After collection, the COCs were washed twice in the collection medium and once in

the maturation medium. The maturation medium consisted of TCM 199 + 0.1% PVA + 1 μl/ml FSH/LH (Folltropin V; Vetrepharm Canada Inc., Belleville, Ont., Canada) + 0.75% kanamycin + 10% (v/v) fetal calf serum (FCS; Gibco BRL, Burlington, Ont., Canada). COCs were matured for 22 h in a drop of 20 ml of maturation medium in a petri dish previously conditioned under tissue culture oil at 38.5 °C, 100% humidity and 5% CO_2. A few hours before the end of maturation, semen for IVF was prepared. Semen from a Charolais bull, known from previous use to be effective for IVF, was used in the present study. Two 0.5 ml straws were thawed in a water bath at 37 °C for 1 min and assessed for post-thaw motility. The semen was pooled in a 15 ml polypropylene test tube (VWR Canlab, Mississagua, Ont., Canada) previously warmed to 37 °C and then dispensed into four 1.7 ml Eppendorf tubes (250 ml of semen each) containing 1 ml of TALP medium. The tubes were placed for 1 h in the incubator at 39 °C to allow selection by swim-up of the sperm. The supernatant containing the motile fraction of sperm was collected from each Eppendorf tube and pooled in a warmed 15 ml Falcon tube. The semen was washed twice by adding 5 ml of TALP medium, followed by centrifugation (500 × *g* for 8 min); the supernatant was discarded and replaced each time. A sample of 5 ml of semen solution was diluted 1:20 with H_2O and sperm concentration was estimated using a modified Neubauer chamber under a phase-contrast microscope (Diaphot; Nikon Canada Inc., Missisauga, Ont., Canada) at 400×. The concentration of spermatozoa in the fertilization solution was adjusted to 3.5 million cells/ml. Heparin was added to the sperm suspension at a final concentration of 10 μg/ml. Fertilization microdroplets of 15 ml were prepared in a petri dish and covered with conditioned tissue culture oil. Matured COCs were washed twice in FERT-TALP medium, and then placed in the sperm suspension in an incubator at 38.5 °C for 20 h.

After exposure to the sperm suspension, the presumptive zygotes were washed once in TALP medium, vortexed for 3 min to remove the remaining cumulus cells, and washed again in IVC medium before being transferred to conditioned IVC microdroplets under tissue culture oil. The IVC medium was CR1 + 10% (v/v) FCS. The developing zygotes were maintained at 38.5 °C, in an atmosphere of 5% CO_2 and 100% humidity, and evaluated for development at the 2- to 4-cell, 8- to 16-cell, morula, and blastocyst stages (i.e. at 36, 84, 120 and 192 h post-insemination). Regardless of the developmental stage attained, culture was terminated on Day 9 (216 h post-insemination).

2.4. Statistical analyses

In vitro development of oocytes (i.e. proportion that developed to the cleavage, 8- to 16-cell, morula or blastocyst stages) was compared among the four follicular phases (Day 2, 3, 5 or 7) and among the three morphological categories (denuded, degenerated or healthy) by Chi-squared analyses. For analyses involving the local effects of the CL and dominant follicle, the ovary was considered the experimental unit (*n*). Within each day-group, animals were ranked according to the number of blastocysts they produced. The highest four producers were randomly assigned to one of four subgroups; the next highest producers were similarly assigned, and so forth until all cows were placed in one of the four subgroups. This was done in an attempt to overcome the intrinsic limitation of in vitro embryo production when done with few oocytes per culture droplet, and when a number of

the ovaries did not produce any embryos because of a small number of COC collected (e.g. one to four oocytes per ovary). Once formed, the subgroups were retained for all successive statistical analyses. Using a mean and variance derived from each of the subgroups, two-way ANOVA were used to examine the local effects (ipsilateral versus contralateral) of the CL or the dominant follicle on blastocyst production in the respective day-groups. To determine the local effects of ovarian structures (CL or dominant follicle) on oocyte morphology, and its interaction with follicular stage (Day 2, 3, 5 or 7), data were transformed (arcsin of the square root of the proportion of oocytes in a given morphologic category) so that two-way ANOVA could be done. The theoretical binomial error variance used for the ANOVA was $821/n$ (n = number of subgroups). A probability value of <0.05 was considered significant.

3. Results

3.1. COC morphology

The effect of the day of collection on the morphology of COC is presented in Table 1. The percentage of COC classified as healthy ranged from 59.8 to 67.7%, and did not vary among day-groups. The percentage of denuded oocytes decreased ($P < 0.05$) from Day 3 (16%) to Days 5 (5%) and 7 (3%). Conversely, the percentage of degenerate COC increased ($P < 0.05$) from Day 3 (19%) to Days 5 (35%) and 7 (29%).

No differences were detected in the proportion of either healthy or degenerate oocytes collected from the ovary containing the dominant follicle or from the contralateral ovary on different days of the follicular wave. However, fewer ($P < 0.0001$) denuded oocytes were collected from the ovary containing the CL than from the contralateral ovary. Moreover, the proportion of denuded oocytes collected from the ovary containing the dominant follicle was higher than from the contralateral ovary ($P = 0.03$). An interaction ($P < 0.0001$) between CL-side and day-group resulted from fewer denuded oocytes collected on Day 7 from the ovary containing the CL compared to the ovary contralateral to the CL. Similarly, an interaction ($P = 0.03$) between side of the dominant follicle and day resulted from a greater proportion of denuded oocytes collected on Day 7 from the ovary containing the dominant follicle compared to the contralateral ovary.

Table 1
Morphology of COC collected from subordinate follicles (≥3 mm) on successive days after follicular wave emergence (Day 0)

Day	Oocytes	Denuded (%)	Degenerate (%)	Healthy (%)
2	365	40 (11.0)^{a}	109 (29.9)^{a,b}	216 (59.2)^{a}
3	319	52 (16.3)^{a,c}	61 (19.1)^{b}	206 (64.6)^{a}
5	436	23 (5.3)^{a,b,c}	151 (34.6)^{a}	262 (60.1)^{a}
7	189	6 (3.2)^{b}	55 (29.1)^{a,b}	128 (67.7)^{a}
Total	1309	121 (9.2)	376 (28.7)	812 (62.0)

Within columns, values without a common superscript (a, b, c) are different ($P < 0.05$).

Table 2
In vitro development of oocytes collected from subordinate follicles (≥3 mm) at successive stages after follicular wave emergence (Day 0)

Day	IVC[1]	No development (%)	2 -to 4-cell (%)	8- to 16-cell (%)	Morula (%)	Blastocyst (%)
2	138	47 (34.0)^{b,c}	91 (66.0)^{a,b}	72 (52.2)^{a}	37 (26.8)^{b}	17 (12.3)^{b}
3	116	34 (29.3)^{c}	82 (70.7)^{a}	43 (37.0)^{b}	30 (25.8)^{b}	15 (12.9)^{b}
5	216	94 (43.5)^{a,b}	122 (56.5)^{b,c}	96 (44.4)^{a,b}	83 (38.4)^{a}	49 (22.7)^{a}
7	163	80 (49.0)^{a}	83 (51.0)^{c}	57 (34.9)^{b}	44 (27.0)^{b}	26 (15.9)^{b}

Within columns, values without a common superscripts (a, b, c) are different ($P < 0.05$).
[1] All COCs, regardless of morphology, were matured, fertilized and cultured. Values at each stage of development are calculated as a percentage of the total number of oocytes undergoing IVC.

Table 3
In vitro development of oocytes collected from subordinate follicles (≥3 mm) at successive stages after follicular wave emergence (Day 0)

Day	IVC	No development (%)	2- to 4-cell[1]	8- to 16-cell[1]	Morula[1]	Blastocyst[1]
2	138	47 (34)^{b,c}	91 (66)^{a,b}	72 (79)^{a,b}	37 (51.4)^{b}	17 (46)^{a}
3	116	34 (29.3)^{c}	82 (70.7)^{a}	43 (52.4)^{b}	30 (69.7)^{a}	15 (50)^{a}
5	216	94 (43.5)^{a,b}	122 (56.5)^{b,c}	96 (78.6)^{a}	83 (86.5)^{a}	49 (59)^{a}
7	163	80 (49)^{a}	83 (51)^{c}	57 (68.6)^{a}	44 (77.2)^{a}	26 (59)^{a}

Within columns, values without a common superscript (a, b, c) are different ($P < 0.05$).
[1] Values are calculated as percentages of the previous stage of development.

3.2. *In vitro development*

Technical problems with the in vitro culture procedures were experienced during the first three replicates. Therefore, the following results were obtained from the last five replicates (74 cows). The effect of day of the follicular wave on oocyte competence is presented in Tables 2 and 3. A greater percentage ($P < 0.05$) of oocytes collected on Day 5 of the follicular wave developed to the morula and blastocyst stages than in any of the other day-groups (Table 2). The percentage of oocytes that developed to the 2- to 4-cell stage was lower ($P < 0.05$) in the Day 7 group than in the Day 2 and 3 groups.

The mean number of oocytes that developed to the blastocyst stage per ovary was not affected by the local presence of the CL ($P = 0.9$) or the dominant follicle ($P = 0.14$). Consistent with the analysis in Table 2, more ($P < 0.05$) blastocysts were produced from oocytes that were collected on Day 5 than from any other day-group, regardless of the location of the CL or dominant follicle.

4. Discussion

Developmental competence of oocytes is of paramount importance in determining the success of an in vitro embryo production protocol [17]. There is increasing evidence that

competence is acquired by the oocytes while they are still in the follicle [18]. In an attempt to develop criteria that are predictive of oocyte quality (competence), follicle dimension has been studied. Oocytes contained in follicles <2 mm in diameter have been reported to have very low developmental ability [7], while follicles between 3 and 8 mm in diameter contained oocytes with similar developmental ability [7,8,19]. These findings suggested that the use of follicle dimension was a good predictor of oocyte competence. However, the degree of variation in follicle dimension observed in ovaries during the first 8 days of the estrous cycle in the present study did not change dramatically. If follicle dimension had been the criterion for selecting oocytes, we would have been unable to distinguish between different functional stages of follicular development. Elucidation of the wave-like pattern of follicle development in cattle [12] has indicated that follicles with the same diameter can be in very different physiological stages of development, from growing to static to atretic. Results of this study show that the developmental competence of oocytes is related to the status of the follicle from which it was obtained, regardless of its dimensions.

Day 5 after follicular wave emergence may be the most appropriate time for oocyte collection from subordinate follicles for optimal in vitro embryo production in cattle. This time corresponds to the stage of development in which most of the subordinate follicles are in late static or early atretic phases [12]. These data confirm those of a previous study [20] in which a higher percentage of expanded and hatched blastocysts were produced from oocytes collected during the static or regressing phases of the follicular wave compared to oocytes collected from follicles in the growing phase. Results of these studies are also consistent with another study [18] in which a higher percentage of blastocysts was obtained when ovaries were left in saline solution at 30 °C for 4 h after slaughter, prior to COC collection. It appears that the effects of early follicular atresia are beneficial to oocyte competence, but the reasons for this phenomenon are still unknown.

Maternal mRNA drives early stages of embryonic development until the embryonic genome takes over [21]. Oocytes that are maintained in the follicular environment for a longer period of time may accumulate a greater amount of, or may be more efficient in accumulating, maternal mRNA in the cytoplasm. When the oocyte is removed from the follicle, it loses the ability to produce RNA in less than 2 h [22]. Insufficient cytoplasmic maturation of the oocyte may lead to an incorrect switch from maternal to embryonic control of development and, as a result, development failure. This is consistent with the observation that the effects of improper maturation are mainly seen later in development rather than during IVM or IVF [23]. Prolonged metabolic coupling between the oocyte and the granulosa cells of the cumulus oophorus may play an important role in this regard. In vivo, gap junctions between the two cellular compartments remain patent until after the preovulatory LH surge [24]. However, the process of gap junction breakdown, as assessed by Connexin 43 rearrangement in intracellular clusters, began immediately upon removal of the COC from the follicular environment and was complete after 6 h of IVM [25]. A longer sojourn within the follicle may permit greater storage of maternal mRNA and proteins within the oocyte, making the oocyte more capable of developing to the blastocyst stage. Further, germinal vesicle breakdown was initiated in subordinate follicles in the late static and early regressing phases, without the preovulatory surge of LH [20], and oocytes of this phase were associated with better embryo development than were oocytes of earlier phases.

Asymmetry in follicular dynamics between the left and right ovaries of the same individual has been the subject of many studies, and has been used to elucidate local versus systemic mechanisms that control ovarian function [12]. Some have reported greater follicular activity in the right ovary in cattle and a higher incidence of right-side ovulations (ca. 60%; [26]), whereas others report no such differences [27,28]. Ginther et al. [27] concluded that the dominant follicle effects follicle suppression by systemic rather than local channels, and results of the present study are consistent with these findings. A positive intra-ovarian effect of the CL on the development of small antral follicles ($\leq$3 mm) has been documented in cattle [26]. However, no differences in the developmental competence of oocytes collected from follicles in the presence or absence of the CL were found in the present study. These findings have important implications regarding mechanisms controlling ovarian function and the roles of locally produced ovarian peptides. It appears that the two ovaries act primarily as a single unit, and follicular counterparts between ovaries influence each other through systemic rather than local routes.

Selection of bovine oocytes for IVM on the basis of visual assessment of morphological features was first examined by Leibfried and First [29]. Since then, many reports have proposed classification schemes based on the compactness and number of layers of cumulus cells surrounding the oocyte [30–32] and on the appearance of the oocyte itself [33,34]. Oocytes with the highest developmental competence have been reported to have an even, smooth, finely granulated cytoplasm, and are surrounded by less than three compact layers of cumulus cells. A positive correlation between COC morphology and blastocyst yield after in vitro culture has been reported [35]. We expected a greater proportion of oocytes with a compacted cumulus in the phase of the follicular wave that resulted in the highest blastocyst yield (i.e. Day 5). However, the percentage of morphologically healthy COC did not change over time during the follicular wave. Conversely, the percentage of denuded oocytes decreased from Day 3 to Days 5 and 7, while the percentage of degenerate COC increased from Day 3 to Days 5 and 7. In a previous study [20], a higher percentage of expanded COCs were collected from follicles in the regressing phase of the wave. This is in accordance with the results of another report in which oocytes with the highest competence were those surrounded by a less compact cumulus [5]. In the present study, oocytes with expanded cumulus cell layers were assigned to the degenerate group, acknowledging the custom of in vitro embryo production techniques of considering only oocytes with compacted cumulus as healthy and viable for IVM. However, the relationship between follicle degeneration, cumulus expansion and oocyte competence shown in this study should serve as a caveat for re-evaluating the current morphologic definition of what is considered a degenerate or healthy oocyte. These results may form a new basis for revising the criteria for selecting oocytes for in vitro embryo production in the cow.

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References

[1] Goto K, Kajihara Y, Kosaka S, Koba M, Nakanishi Y, Ogawa K. Pregnancies after co-culture of cumulus cells with bovine embryos derived from in vitro fertilization of in vitro matured follicular oocytes. J Reprod Fertil 1988;83:753–8.

[2] Hanada A, Enya Y, Suzuki T. Birth of calves by non-surgical transfer of in vitro fertilized embryos obtained from oocytes matured in vitro. Jpn J Anim Reprod 1986;32:208.

[3] Lu KH, Gordon I, Chen HB, McGovern H. In vitro culture of early bovine embryos derived from in vitro fertilization of follicular oocytes matured in vitro. In: Proceedings of the Third Scientific Meeting of the European Embryo Transfer Association, vol. 70, Lyon, France; 1987.

[4] Farin PW, Crosier AE, Farin CE. Influence of in vitro systems on embryo survival and fetal development in cattle. Theriogenology 2001;55:151–70.

[5] Blondin P, Sirard MA. Oocyte and follicular morphology as determining characteristics for developmental competence in bovine oocytes. Mol Reprod Dev 1995;41:54–62.

[6] Bracket B, Zuelke K. Analysis of factors involved in the in vitro production of bovine embryos. Theriogenology 1993;39:43–64.

[7] Pavlok A, Lucas-Hahn A, Niemann H. Fertilization and developmental competence of bovine oocytes derived from different categories of antral follicles. Mol Reprod Dev 1992;31:63–7.

[8] Tan SJ, Lu KH. Effect of different estrous stages of ovaries and sizes of follicles on generation of bovine embryos in vitro. Theriogenology 1990;33:335.

[9] Ginther OJ, Knopf L, Kastelic JP. Temporal association among ovarian events in cattle during oestrous cycles with two and three follicular waves. J Reprod Fertil 1989;87:223–30.

[10] Ginther OJ, Knopf L, Kastelic JP. Ovarian follicular dynamics in heifers during early pregnancy. Theriogenology 1989;41:247–54.

[11] Knopf L, Kastelic JP, Schallenberger E, Ginther OJ. Ovarian follicular dynamics in heifers: test of two-wave hypothesis by ultrasonically monitoring individual follicles. Domest Anim Endocrinol 1989;6: 111–9.

[12] Adams GP. Comparative pattern of follicle development and selection in ruminants. J Reprod Fertil Suppl 1999;54:17–32.

[13] Adams GP, Matteri RL, Kastelic JP, Ko JCH, Ginther OJ. Association between surges of follicle-stimulation hormone and the emergence of follicular waves in heifers. J Reprod Fertil 1992;94:177–88.

[14] Ko JCH, Kastelic JP, Ginther OJ. Effect of dominant follicle on ovarian follicular dynamics during the estrous cycle in heifers. J Reprod Fertil 1991;91:511–9.

[15] Bergfelt DR, Lightfoot KC, Adams GP. Ovarian synchronization following ultrasound-guided transvaginal follicle ablation in heifers. Theriogenology 1994;42:895–907.

[16] Gibbons JR, Wiltbank MC, Ginther OJ. Functional interrelationships between follicles greater than 4 mm and the follicle-stimulating hormone surge in heifers. Biol Reprod 1997;57:1066–73.

[17] Sirard MA, Blondin P. Oocyte maturation and IVF in cattle. Theriogenology 1996;42:417–26.

[18] Blondin P, Coenen K, Guilbault LA, Sirard MA. In vitro production of bovine embryos: developmental competence is acquired before maturation. Theriogenology 1997;47:1061–75.

[19] Lonergan P, Monaghan P, Rizos D, Boland MP, Gordon I. Effect of follicle size on bovine oocyte quality and developmental competence following maturation, fertilization and culture in vitro. Mol Reprod Dev 1994;37:48–53.

[20] Salamone DF, Adams GP, Mapletoft RJ. Changes in the cumulus oocyte complex of subordinate follicles relative to follicular wave status in cattle. Theriogenology 1999;52:549–61.

[21] Latham KE. Mechanisms and control of embryonic genome activation in mammalian embryos. Int Rev Cytol 1999;193:71–124.

[22] Sirard MA, Coenen K. The effect of hormones during in vitro meiotic inhibition with cycloheximide on subsequent development of bovine oocytes. Biol Reprod 1994;50:145 [abstract].

[23] Leibfried-Rutledge L, Critser ES, Eyestone WH, Northey DL, First NL. Developmental potential of bovine oocytes matured in vitro and in vivo. Biol Reprod 1987;36:376–83.

[24] Larsen WJ, Wert SE, Brunner GD. A dramatic loss of gap junctions is correlated with germinal vesicle breakdown in rat oocytes. Dev Biol 1986;113:517–21.

[25] Sutovsky P, Flechon JE, Flechon B, Motlik J, Peynot N, Chesne P, et al. Dynamic changes of gap junctions and cytoskeleton during in vitro culture of cattle oocyte cumulus complexes. Biol Reprod 1993;49: 1277–87.
[26] Pierson RA, Ginther OJ. Follicular populations during the estrous cycle in heifers. II. Influence of right and left sides and intraovarian effect of the corpus luteum. Anim Reprod Sci 1987;14:177–86.
[27] Ginther OJ, Kastelic JP, Knopf L. Intraovarian relationships among dominant and subordinate follicles and the corpus luteum in heifers. Theriogenology 1989;32:787–95.
[28] Sirois J, Fortune JE. Ovarian follicular dynamics during the estrous cycle in heifers monitored by real-time ultrasonography. Biol Reprod 1988;39:308–17.
[29] Leibfried L, First NL. Characterization of bovine follicular oocytes and their ability to mature in vitro. J Anim Sci 1979;48:76–86.
[30] Hazeleger NL, Stubbings RB. Developmental potential of selected bovine oocyte cumulus complexes. Theriogenology 1992;37:219.
[31] Laurincik J, Kroslak P, Hyttel P, Pivko J, Sirotkin AV. Bovine cumulus expansion and corona-oocyte disconnection during culture in vitro. Reprod Nutr Dev 1992;32:151–61.
[32] Yang YB, Lu KH. The influence of bovine oocyte type on in vitro fertilization and subsequent in vitro development. Theriogenology 1990;33:355.
[33] de Loos FAM, van Vliet C, van Maurik P, Kruip ThAM. Morphology of immature bovine oocytes. Gamete Res 1989;24:197–204.
[34] Younis AI, Brackett BG, Fayrer-Hosken RA. Influence of serum and hormones on bovine oocyte maturation and fertilization in vitro. Gamete Res 1989;23:189–201.
[35] Hazeleger NL, Hill DJ, Stubbings RB, Walton JS. Relationship of morphology and follicular fluid environment of bovine oocytes to their developmental potential in vitro. Theriogenology 1995;43:509–22.

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Ultrasound image characteristics of ovarian follicles in relation to oocyte competence and follicular status in cattle

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Abstract

Assessment of the quality of the female gamete has become paramount for in vitro procedures. There is a need to identify reliable indicators of oocyte competence and develop a simple, non-invasive method to assess competence. The aim of this study was to investigate the relationships among ultrasonographic attributes of a follicle, its stage of development and the competence of the oocyte that it contains. We tested the hypotheses that follicular echotexture characteristics are related to: (1) the phase of development of the follicle, (2) the presence of the corpus luteum (CL) and/or the dominant follicle in the ovary, and (3) developmental competence of cumulus oocyte complexes (COC) from the same ovary. Crossbred beef cows ($n = 143$), age 4–14 years, were given a luteolytic dose of dinoprost to cause ovulation. Ultrasound-guided ablation of all follicles $\geq$4 mm was done 8 days later to induce new follicular wave emergence during a luteal phase. Ultrasonographic images of dominant follicles and the three largest subordinate follicles ($n = 402$ follicles; 84 cows) were acquired on Days 2, 3, 5 or 7 of the follicular wave (Day 0: wave emergence), i.e. growing, early-static, late static, and regressing phases of subordinate follicle development, respectively. From a subset of these animals ($n = 33$), ovaries were collected within 30 min of slaughter and COC from subordinate follicles $\geq$3 mm underwent in vitro maturation, fertilization and culture to the blastocyst stage.

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Image analysis revealed differences in echotexture between dominant and subordinate follicles among Days 2–7 of the follicular wave. Images of dominant and subordinate follicles at Day 7 of the wave displayed consistently lower grey-scale values ($P < 0.05$) in the peripheral antrum, follicular wall and perifollicular stroma than all other days. Follicle images displayed a consistent pattern of variation in echotexture among follicular phases. Data did not support the hypothesis of a local effect of the CL or dominant follicle on follicular echotexture. Echotexture values of the perifollicular stroma were lower in ovaries that did not produce embryos compared to ovaries that produced embryos. Our results showed that the changes in follicular image attributes are consistent with changes in follicular status. The sensitivity of the technique is not yet sufficient for use in a diagnostic setting, but results provide rationale for further development of image analysis as a tool for evaluating oocyte competence in situ.
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Keywords: Cattle; Echotexture analysis; Follicular dynamics; Image analysis; Oocyte competence; Ovary; Ultrasonography

1. Introduction

The pattern of periodic emergence of ovarian follicular waves in ruminants is regulated by a series of tightly timed systemic feedback mechanisms between the ovary and the pituitary gland (reviewed in Adams, 1999). The results of extensive ultrasonographic studies have documented that follicular growth during the estrous cycle in the cow is characterized by two or three follicular waves (Pierson and Ginther, 1987, 1988; Savio et al., 1988; Sirois and Fortune, 1988; Ginther et al., 1989a,b,c; Knopf et al., 1989). A cohort of follicles begins to grow beyond 4 mm in diameter during each wave. Each follicular wave is preceded by a surge in FSH (Adams et al., 1992). One follicle of the cohort is selected to continue growth (dominant) while other follicles (subordinates) become committed to atresia around Day 5 of the follicular wave.

Oocyte competence has been defined as the ability of oocytes to develop in vitro to the blastocyst stage (Farin et al., 2001) and many attempts have been made to identify indicators of an oocyte's ability to produce a blastocyst (Madison et al., 1992; Hazeleger et al., 1994; Gandolfi et al., 1997). However, current in vitro embryo production protocols use follicle size as the main criterion for selection of oocytes (Lonergan et al., 1994). Subordinate follicles are primary source of oocytes for in vitro embryo production protocols because they are numerous, display a certain degree of developmental ability, and are able to develop into full-term offsprings when transferred into a recipient after in vitro maturation, fertilization and culture. However, follicles of similar diameter may be in very different physiologic phases (i.e. growing, static or regressing) and therefore contain oocytes that may vary considerably in maturational state or developmental competence. The results of recent studies have been interpreted to mean that cumulus oocyte complexes (COC) collected from subordinate follicles in the late static or early regressing phase (Day 5 of the follicular wave) were more competent than those of follicles at other phases, irrespective of follicle dimension (Salamone et al., 1999; Vassena, 2001).

Ultrasound images are composed of a two-dimensional matrix of picture elements (pixels) that differ in their grey-scale value (Kremkau, 1998; Ginther, 1995; Pierson and Adams,

1995). Each pixel is described by one of 256 shades of grey (0 corresponding to black and 255 to white), and represents a discrete tissue reflector (Pierson and Adams, 1995). The ultrasonographic appearance or image pattern of a tissue is referred to as echotexture and is determined by the histologic structure of the tissue (Ginther, 1995; Singh et al., 1997, 1998). Computer algorithms specifically designed for ultrasound image analysis have been developed to overcome the inconsistencies of visual evaluation, and to provide a quantitative approach to grey-scale pixel value analysis (Synergyne©, Version 2.8, WHIRL, Saskatoon, Sask., Canada). These algorithms have been used extensively in studies characterizing the echotexture dynamics of ovarian structures at different phases of the follicular wave (Pierson and Adams, 1995; Singh et al., 1997, 1998; Tom et al., 1998a,b). Phase-specific changes in dominant and subordinate follicles and the corpus luteum (CL) have been characterized by computer-assisted examination of ultrasound image attributes. However, this approach has not been used to assess the developmental competence of oocytes contained within the follicles analyzed.

Local paracrine and autocrine influences exerted by the follicles and corpus luteum have been reported in many in vitro studies (reviewed by Chun and Hsueh, 1998; Albertini et al., 2001; Findlay et al., 2001) and receptors for progesterone and estrogen have been detected in the bovine ovary (Berisha et al., 2002). However, results of in vivo ultrasound studies involving assessment of follicle development and ovulation in relation to the relative position of CL or dominant follicle are not consistent with a local effect (Ginther et al., 1989c; Adams, 1999). Consistent changes in ultrasound image attributes have been associated with the physical and endocrine status of ovarian follicles (Singh et al., 1998). The design of this study allowed us to examine the local effects of the CL and dominant follicle on ultrasound image attributes of subordinate follicles.

The aim of this study was to investigate the relationship between ultrasonographic attributes of a follicle, its stage of development and the competence of the oocyte that it contains. The experimental design allowed us to test the hypotheses that follicular echotexture characteristics are related to: (1) the phase of development of the follicle, (2) the presence of CL and/or the dominant follicle in the ovary, and (3) the developmental competence of COC from the same ovary.

2. Materials and methods

2.1. Animal grouping and image acquisition

Non-pregnant crossbred beef cows 4–14 years of age, were kept in three outdoor paddocks on a private farm, and maintained on a rising plane of nutrition (barley and hay) in preparation for slaughter during July, August and September. Cows with a corpus luteum ($n = 143$) were assigned to one of the four groups to be slaughtered on Day 2 ($n = 41$), Day 3 ($n = 42$), Day 5 ($n = 40$) or Day 7 ($n = 20$) of the follicular wave (Day 0: day of wave emergence). Emergence of a new follicular wave was synchronized among animals to permit ultrasound imag[illegible]gitization and oocyte retrieval on a given day of slaughter. Moreover, the synchronization scheme allowed the induction of a follicular wave during the luteal phase in all animals. Synchronization was done by administering

luteolytic dose of $PGF_{2\alpha}$ analogue (dinoprost; 5 ml Lutalyse (i.m.); Pharmacia & Upjohn Animal Health, Orangeville, Canada) followed 8 days later by transvaginal ultrasound-guided ablation of all follicles ≥4 mm. The emergence of a new follicular wave (Day 0) was expected to occur 1 day after follicle ablation (Bergfelt et al., 1994). The farm and abattoir were distant from the laboratory (255 km) so not more than 20 cows could be processed on a given day because of logistical constraints (i.e. animal treatments and follicle ablations, in vivo ultrasound image capture, slaughter schedule, ovary and oocyte collection, in vitro oocyte procedures). Hence, cows were scheduled to be slaughtered on one of the eight occasions (10–20 cows each) such that two Day-groups were represented on each day of slaughter. The ovaries were examined by transvaginal ultrasonography (Aloka SSD-900; ISM Inc., St. Laurent, Canada) using a 7.5 MHz linear-array transducer 2–3 h before slaughter. The transducer scanning head was mounted on a rigid plastic extension for transvaginal use. Images of the dominant follicle and the three largest subordinate follicles in each ovary were digitized directly from the ultrasound machine to a computer using a video capture board (DTV 2000, Diamond Multimedia Systems Inc., San Jose, CA, USA) and custom-developed software optimized for ultrasound images under the Linux operating system (Agyne©, Version 1.2, WHIRL, Saskatoon, Sask., Canada). From a subset of these animals (n = 33 cows), COC were collected within 30 min after slaughter from subordinate follicles on Days 2, 3, 5 and 7 of follicular wave for in vitro embryo production.

2.2. *In vitro embryo production*

At the abattoir, each ovary was placed in an individual plastic bag containing phosphate-buffered saline (PBS) + 1% (w/v) antibiotic/antimycotic (10,000 IU penicillin, 10 mg streptomycin and 25 μg amphotericin B/ml; Sigma–Aldrich Canada Ltd., Oakville, Ont., Canada). Each bag was marked with an identification number different from the ear tag number of the cow so that the investigator was blinded from Day-groupings. The ovaries were transferred to the laboratory at 30 °C within 3 h of collection. COC were aspirated into a 15 ml vial containing 3 ml of collection medium (TCM 199 + 0.75% kanamycin). Aspiration was accomplished with an 18 g needle at a continuous controlled flow-rate of 35 ml/min. The needle was rinsed before and after aspiration of each ovary. The dominant follicle and, when in doubt, the largest subordinate follicle were aspirated separately from the remaining subordinates. COC from all subordinate follicles ≥3 mm in the same ovary were collected and pooled in the same tube.

The maturation medium consisted of TCM 199 + 0.1% PVA + 1 μl/ml FSH/LH (Folltropin V, Vetrepharm Canada Inc., Belleville, Ont., Canada) + 0.75% kanamycin + 10% (v/v) fetal calf serum (FCS, Gibco BRL, Burlington, Ont., Canada). COC recovered from each ovary were matured for 22 h in a single drop (20 μl) of maturation medium under tissue culture oil at 38.5 °C, 100% humidity and 5% CO_2.

Frozen semen from a single bull, which had been previously used successfully for IVF, was used. A few hours before the end of oocyte maturation, two straws of semen (0.5 ml each) were thawed in a water bath at 37 °C for 1 min, pooled in a pre-warmed conical tube (VWR Canlab, Mississagua, Ont., Canada), and dispensed into 4 × 1.7 ml Eppendorf tubes (250 μl each) containing 1 ml of modified Tyrode's solution containing albumin, lactate

and pyruvate (TALP; Bavister and Yanagimachi, 1977; Bavister et al., 1983) medium each. The tubes were incubated for 1 h at 39 °C to allow swim-up of the sperm. The supernatant (800 μl) was collected from each Eppendorf tube and pooled in a warmed 15 ml tube. The semen was washed in TALP medium and centrifuged (500 G for 8 min) twice. Spermatozoa concentration was adjusted to 3.5 million/ml in the fertilization solution (Bavister and Yanagimachi, 1977; Bavister et al., 1983). Heparin was added to a final concentration of 10 μg/ml. Microdroplets of 15 μl were prepared under tissue culture oil. Matured COC were washed twice in TALP medium, and placed in the sperm suspension for 20 h at 38.5 °C, 100% humidity and ambient air with 5% CO_2.

Presumptive zygotes were washed in TALP medium, vortexed for 3 min, washed in culture medium (CR1 + 10% FCS; Rosenkrantz et al., 1990) and transferred to microdroplets of culture medium under tissue culture oil. The developing zygotes were maintained at 38.5 °C in an atmosphere of 5% CO_2, 95% ambient air and 100% humidity, and subsequently evaluated for development at the 2–4 cell, 8–16 cell, morula, and blastocyst stages (i.e. at 36, 84, 120 and 192 h post-insemination). Regardless of the developmental stage attained, culture was terminated at Day 9 (216 h post-insemination).

2.3. *Quantitative echotexture analysis of ultrasound images*

Analysis of ultrasound images was performed using a series of custom-developed computer algorithms optimized for ultrasonography (Synergyne©, Version 2.8, WHIRL, Saskatoon, Sask., Canada) on a Sun Sparc Station 20 (Sun Microsystems, Mt. View, CA, USA) computer. A total of 746 images from 143 cows were initially digitized. Due to technical problems with ultrasound machine settings and image digitization, images from 59 cows were not included in the analysis. Computer-assisted analyses were therefore performed on 402 follicle images from 84 cows; 15 cows on Day 2 ($n = 92$ images), 23 cows on Day 3 ($n = 95$ images), 27 cows on Day 5 ($n = 132$ images), and 19 cows on Day 7 ($n = 83$ images). Image analyses were performed by an individual to whom identities of individual images and cows were not disclosed.

2.3.1. *Spot analysis of the follicle antrum*

Quantitative echotexture analysis of the follicular antrum was performed by placing a computer-generated circular spot at the center of the antral region of each follicle. A computer-generated circular spot was placed over the image of the follicle antrum. The diameter of the spot was enlarged progressively by increments of two pixel units until the follicular wall was encountered—apparent by a sudden increase in mean values at the antrum–follicular wall interface. The spot diameter was then decreased by two pixel units and values were recorded. This technique was used to ensure that only the antrum was included in analysis of less than perfectly spherical follicles. The mean grey-scale value (average of values for all pixels within the measuring spot; black: 0, white: 255), heterogeneity (standard deviation of values of all pixels within the measuring spot), and the diameter of the spot were recorded (Singh et al., 1998).

To determine if image data required normalization among days prior to statistical analysis (Singh et al., 1998), normalized and non-normalized data from a subset of 40 images were compared. No differences were detected in mean grey-scale values ($P < 0.05$) or

heterogeneity ($P < 0.05$) between normalized and non-normalized data sets; hence, image normalization was not performed in this study.

Spot analysis provided one value per follicles for each endpoint. Values for echotexture endpoints (from both spot and line analyses) of the three largest subordinate follicles per ovary were averaged to obtain a single value for each endpoint before statistical analysis. This was done to make direct comparisons with oocyte competence data where COC from subordinate follicles were pooled from each ovary into a single group.

2.3.2. Line analysis of the peripheral antrum, follicle wall and perifollicular stroma

Line analyses were used to measure the grey-scale values of pixels along a straight line traversing the area of interest (Pierson and Adams, 1995; Singh et al., 1998). A computer-generated line was placed on each side of the image of the follicular wall at approximately 2- and 10-O'clock positions; locations selected to minimize the confounding effects of enhanced through transmission and refraction artifacts (Kremkau, 1998; Ginther, 1995). A two-dimensional graph was generated for each line, corresponding to the value of each pixel along the length of the line (i.e. antrum, follicle wall and stroma; Fig. 1). The linear distance represented by one pixel was calculated by measuring the length of the scale bar on the ultrasound image in number of pixels and was estimated to be 0.25 mm. The antrum–wall interface was identified by the first sudden increase in grey-scale value along the computer-generated line (Singh et al., 1998). Based on the results of a morphometric

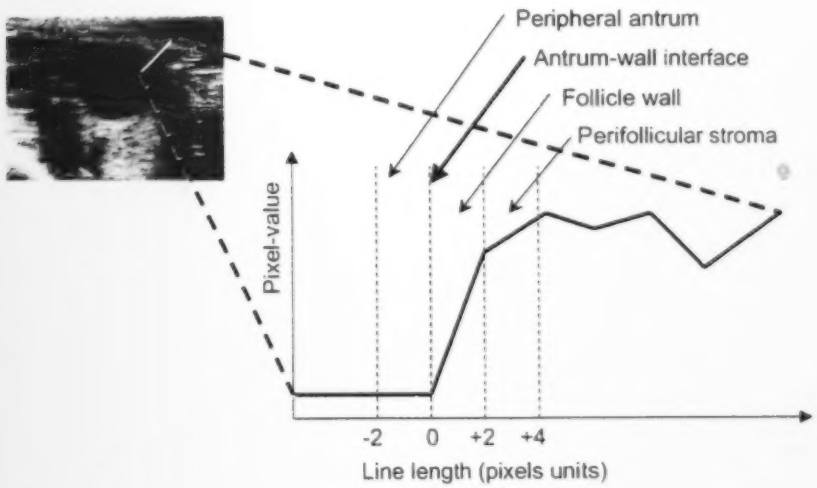

Fig. 1. Graphic representation of grey-scale values of each pixel along a computer-generated line traced over an ultrasound image of the ovarian follicle wall. Dotted vertical lines represent the borders of the areas of interest (i.e. peripheral antrum, follicle wall, perifollicular stroma). The antrum–wall interface is identified as a point along the line where pixel values suddenly increase. This interface was used as a point of reference (pixel number: 0) for defining the areas of interest (i.e. −2 to 0 pixels: peripheral antrum; 0–2 pixels: follicle wall; 2–4 pixels: perifollicular stroma). Values (peak and mean grey-scale value, heterogeneity, and area under the curve) of the segments were recorded separately.

study of bovine follicles (Singh and Adams, 2000), data for only that part of the line extending two pixels inward from the antrum–wall interface (peripheral antrum), two pixels outward from the interface (follicle wall), and two more pixels outward from the follicle wall (perifollicular stroma; Fig. 1) were used for statistical analysis (Singh et al., 1998). Peak grey-scale values, mean grey-scale values, heterogeneity, and area under the curve were recorded for each two pixel segment of the line. The intercept and slope of the best-fit simple linear regression line for each of the three segments (peripheral antrum, follicle wall, perifollicular stroma) were also obtained. The values of the three segments for each follicle were obtained by taking the average of two values (one line per side, two sides per follicle).

2.4. Echotexture analysis and oocyte competence

To study the relationship between the developmental competence of oocytes and the image characteristics of the subordinate follicles, a smaller dataset was used which included only those animals that were used for in vitro embryo production. The echotexture data from a total of 33 animals (Day 2, $n = 3$; Day 3, $n = 4$; Day 5, $n = 11$; Day 7, $n = 15$) were compared with the developmental competence of their oocytes. For this analysis, ovaries were categorized into 3 groups irrespective of the day of the follicular wave on which they were collected. The groups were: (1) ovaries whose oocytes did not develop in vitro, (2) ovaries whose oocytes displayed less than average embryonic development in vitro and, (3) ovaries whose oocytes displayed more than average embryonic development in vitro. An average value of the developmental ability of oocytes from all animals ($n = 33$) on a per ovary basis was calculated as the average of the proportion of oocytes from subordinate follicles that reached the cleavage stage (0.52), the 8–16 cell stage (0.4) and the morula stage (0.33). These values were used to determine whether oocytes from a given ovary had average, less than average or more than average developmental competence.

In order to study the effect of oocyte competence and day of follicular wave on echotexture endpoints, data from Day 2 and Day 3 groups were combined ($n = 7$) in a single group due to the low number of animals ($n = 3$ for Day 2, $n = 4$ for Day 3 group). In this analysis, we distinguished oocytes from subordinate follicles in the growing phase (Days 2–3), static phase (Day 5), and regressing phase (Day 7) of the follicular wave. For each of the embryonic stages (cleavage, 8–16 cell and morula), ovaries from each of the phases (growing, static and regressing) were subcategorized into the same three groups described previously (i.e. no development, less than average and more than average development).

2.5. Statistical analyses

Except for analyses involving local effects of the CL and dominant follicle, the animal was considered the experimental unit. For analyses involving the local effects of the CL and dominant follicle, the ovary was considered the experimental unit (n). For all analyses, a probability value of less than 0.05 was considered significant. Whenever main effects or interaction term were significant ($P < 0.05$), means were compared by the least significant difference method. Echotexture characteristics of the follicles were compared among the four follicular phases (i.e. Days 2, 3, 5, or 7) and between the two follicular categories (i.e. dominant or subordinate) by two-way analysis of variance. To determine the local effects of

ovarian structures (CL or dominant follicle) on the echotexture of subordinate follicles, and its interaction with follicular stage (Days 2, 3, 5 or 7), three-way analyses of variance were performed (model = ipsilateral or contralateral (I/C) to CL, I/C to dominant follicle, day, I/C to CL × day, I/C to dominant follicle × day, I/C to CL × I/C to dominant follicle). Image data for the dominant follicles were analyzed by a separate two-way analysis of variance to study the local effect of CL and the day (model = I/C to CL, day, I/C to CL × day). The relationship between follicular echotexture and the ability of the contained oocytes to develop to the cleavage, 8–16 cell, or morula stages (ovary category) was assessed by two-way analysis of variance (model = ovary category, day, ovary category × day).

3. Results

3.1. Echotexture analysis of follicular antrum (spot analysis)

The effect of day of the follicular wave on the mean grey-scale values of the antrum of dominant and subordinate follicles is presented in Table 1. The overall mean grey-scale values of the antrum of dominant follicles were lower than in subordinate follicles ($P = 0.001$). Regardless of the type of follicle (dominant or subordinate), Day 7 follicles had a lower mean grey-scale value than those on Days 2, 3 or 5 ($P = 0.02$). The interaction between day of the wave and follicle type was not significant ($P = 0.74$). Heterogeneity analysis did not reveal any influence of the day of the follicular wave ($P = 0.54$) or of the type of follicle ($P = 0.14$).

3.2. Echotexture analysis of peripheral antrum, follicle wall and perifollicular stroma (line analysis)

The effect of the phase of the follicular wave on echotexture characteristics of the peripheral antrum, follicular wall and perifollicular stroma of dominant and subordinate follicles is presented in Figs. 2–4. The pattern of change in grey-scale values over days (peak grey-scale values, Fig. 2; area under the curve, Fig. 3; mean grey-scale values, Fig. 4) for the three parts of the follicle was similar for dominant and subordinate follicles. The values for the dominant and subordinate follicles tended to follow a pattern over days, consistently decreasing

Table 1
Grey-scale values (mean ± S.E.M.) of the antrum of ovarian follicles on different days of the follicular wave (Day 0: wave emergence)

Follicle	Day 2	Day 3	Day 5	Day 7	Overall
Dominant	3.9 ± 0.6 (9)	5.0 ± 0.9 (16)	5.1 ± 1.0 (25)	2.1 ± 0.2 (15)	4.2 ± 0.5^{a} (65)
Subordinate	11.3 ± 0.9 (25)	10.0 ± 0.9 (37)	10.1 ± 0.7 (44)	8.1 ± 0.8 (30)	9.8 ± 0.4^{b} (136)
Overall	9.3 ± 0.9^{y} (34)	8.5 ± 0.8^{y} (53)	8.2 ± 0.6^{y} (69)	6.1 ± 0.7^{z} (45)	

Day ($P = 0.019$), follicle ($P < 0.0001$), Day × follicle interaction ($P = 0.74$). Values in parentheses indicate the number of observations. Within columns, values with no common superscripts (a and b) are different ($P < 0.05$); within rows, values with no common superscripts (y and z) are different ($P < 0.05$).

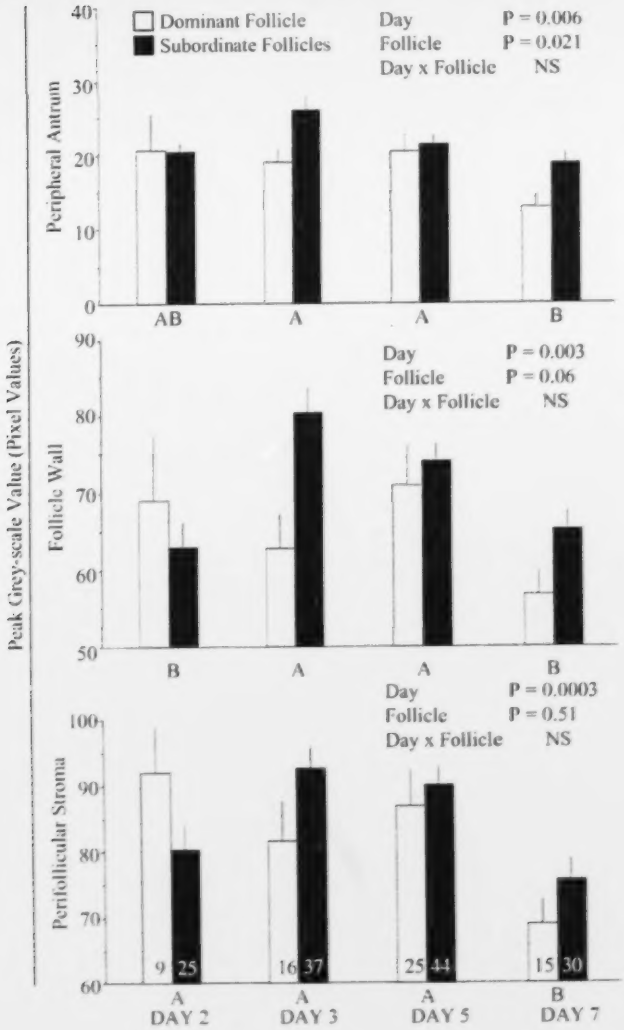

Fig. 2. Peak grey-scale value (mean ± S.E.M.) obtained by line analysis of the peripheral antrum, follicle wall and perifollicular stroma of ultrasound images of dominant (white bars) and subordinate (black bars) follicles in cattle ($n = 84$ cows) on different days of the follicular wave (Day 0: wave emergence). Numbers at the bottom of the bars of the lower chart indicate the number of follicles examined. (A, B) Values (dominant and subordinate combined) with no common letters are different among days ($P < 0.05$).

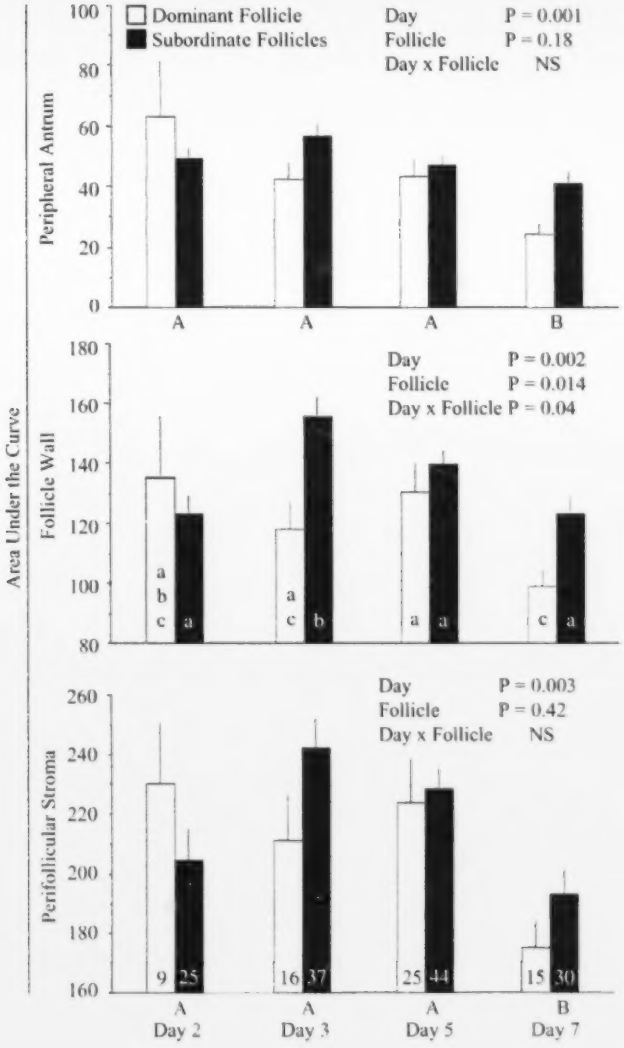

Fig. 3. Area under the curve (pixel^2; mean ± S.E.M.) obtained by line analysis of the peripheral antrum, follicle wall and perifollicular stroma of ultrasound images of dominant (white bars) and subordinate (black bars) follicles in cattle ($n = 84$ cows) on different days of the follicular wave (Day 0: wave emergence). Numbers at the bottom of the bars of the lower chart indicate the number of follicles examined. (A, B) Values (dominant and subordinate combined) with no common letters are different among days ($P < 0.05$). (a, b, c) Bars with no common letters are different among days and follicles ($P < 0.05$).

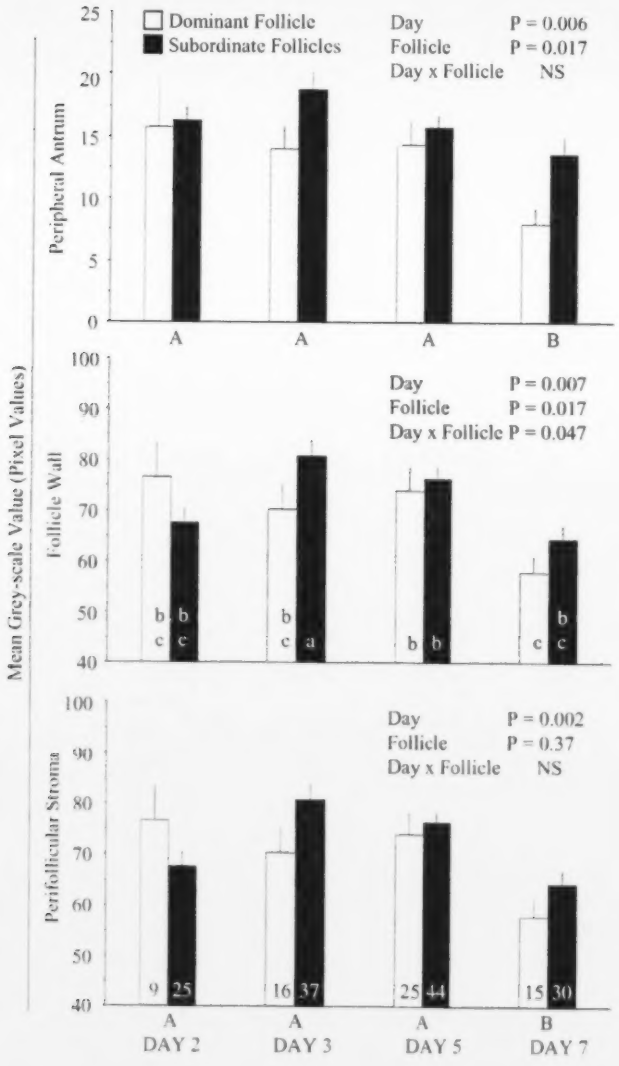
Mean Grey-scale Value (Pixel Values)
Peripheral Antrum
Dominant Follicle
Subordinate Follicles
Day P = 0.006
Follicle P = 0.017
Day x Follicle NS
A A A B
Follicle Wall
Day P = 0.007
Follicle P = 0.017
Day x Follicle P = 0.047
Perifollicular Stroma
Day P = 0.002
Follicle P = 0.37
Day x Follicle NS
9 25 16 37 25 44 15 30
A A A B
DAY 2 DAY 3 DAY 5 DAY 7

Fig. 4. Mean grey-scale value (mean ± S.E.M.) obtained by line analysis of the peripheral antrum, follicle wall and perifollicular stroma of ultrasound images of dominant (white bars) and subordinate (black bars) follicles in cattle (n = 84 cows) on different days of the follicular wave (Day 0: wave emergence). Numbers at the bottom of the bars of the lower chart indicate the number of follicles examined. (A, B) Values (dominant and subordinate combined) with no common letters are different among days ($P < 0.05$). (a, b, c) Bars with no common letters are different among days and follicles ($P < 0.05$).

from Days 5–7 ($P < 0.05$). This pattern was conserved in all follicular segments (peripheral antrum, follicle wall and perifollicular stroma) and for all the echotexture endpoints analyzed (peak grey-scale values, mean grey-scale values and area under the curve). Mean grey-scale value and area under the curve for the wall of subordinate follicles showed an increase ($P < 0.05$) between Days 2 and 3, followed by a decrease in values ($P < 0.05$) between Days 3 and 5. Peripheral antrum and peripheral stroma of the subordinate follicles also showed similar changes in values numerically, albeit statistically non-significant, between Days 2 and 5.

3.3. Echotexture analysis and local effect of ovarian structures

There was no local effect of the dominant follicle or of the CL on any of the subordinate follicle echotexture endpoints studied. Consistent with the previous line analysis, the day effect was significant for peak grey-scale values, heterogeneity and slope of the regression line for the peripheral antrum ($P = 0.001$, $P = 0.0001$ and $P = 0.0001$, respectively), and for peak and mean grey-scale values, and area under the curve for the follicle wall ($P = 0.0001$). There was no local effect of the CL on dominant follicle echotexture endpoints except those involving the peak grey-scale values of the peripheral antrum (Table 2).

3.4. Echotexture analysis and oocyte competence

We compared the echotexture data of subordinate follicles from ovaries of 33 animals (Day 2, $n = 3$; Day 3, $n = 4$; Day 5, $n = 11$; Day 7, $n = 15$) with the developmental competence of the oocytes from the same ovaries irrespective of the day of the follicular wave on which they were collected. Ovaries were categorized in three groups: (1) ovaries whose oocytes did not develop to cleavage stage, 8–16 cell stage or morula stage, (2) ovaries whose oocytes displayed less than average embryonic development, and (3) ovaries whose oocytes displayed more than average embryonic development in vitro. Results are presented in Fig. 5. No differences in any echotexture endpoint of subordinate follicles were found when ovaries were categorized based on no development, less than average or more than average development to the cleavage stage or the morula. For the 8–16 cell stage of embryonic development, however, peak grey-scale values, area under the curve, mean grey-scale values and heterogeneity of the perifollicular stroma differed among different

Table 2
Local effect of the CL on the peak grey-scale value of ultrasound images of the peripheral antrum of dominant follicles in cattle on different days of the follicular wave (Day 0: wave emergence; ipsilateral: dominant follicle in the ovary ipsilateral to the CL; contralateral: dominant follicle in the ovary contralateral to the CL)

	Day 2	Day 3	Day 5	Day 7	Overall
Peak grey-scale					
Ipsilateral	$17.4 \pm 4.1^{a,b}$	$19.4 \pm 4.0^{a,b}$	23.2 ± 3.3^{a}	8.9 ± 2.4^{b}	18.4 ± 2.6
Contralateral	*	$18.5 \pm 2.3^{a,b}$	$19.3 \pm 4.6^{a,b}$	$15.9 \pm 2.5^{a,b}$	19.0 ± 2.1
Overall	20.6 ± 4.9	18.9 ± 2.0	21.5 ± 2.7	12.9 ± 2.0	

(*) Only one observation available; Day ($P = 0.01$), ipsilateral or contralateral to CL ($P = 0.03$), Day × CL interaction ($P = 0.04$). Among cells, values with no common superscripts (a and b) are different ($P < 0.05$).

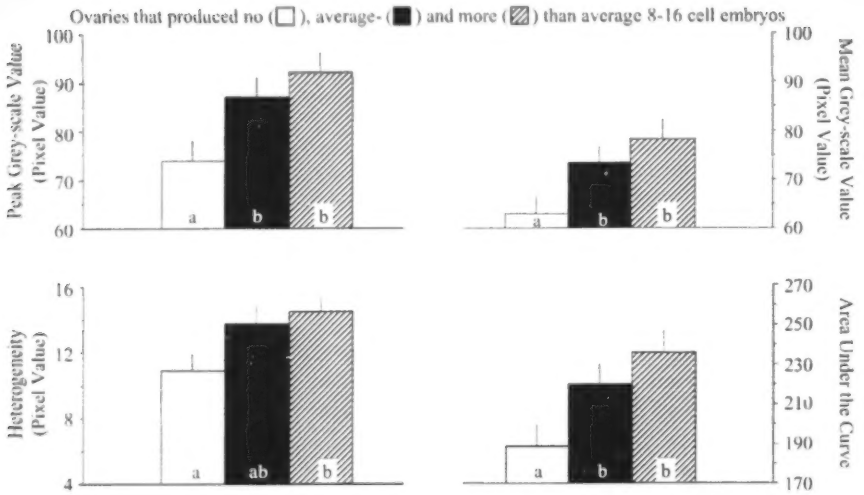

Fig. 5. Peak grey-scale value, mean grey-scale value, heterogeneity and area under the curve (mean ± S.E.M.) of subordinate follicles, obtained by line analysis of perifollicular stroma, from ovaries that produced no (white bars), less than average (black bars) or more than average (grey bars) embryos developing to the 8–16 cell stage. (a, b) Bars values with no common letters are different ($P < 0.05$).

categories of the ovaries. Heterogeneity of the perifollicular stroma of the subordinate follicles was lower for the ovaries that did not produce embryo development compared to those that produced more than average development ($P < 0.05$). The subordinate follicles whose COC produced no embryo development had lower peak grey-scale values, area under the curve and mean grey-scale values ($P < 0.05$) than those that produced 8–16 cell stage embryos, either below or above average.

To study the effect of oocyte competence and day of follicular wave on echotexture endpoints, data from subordinate follicles in the growing phase (Days 2–3), static phase (Day 5), and regressing phase (Day 7) of the follicular wave were examined. For each of the embryonic stages (cleavage, 8–16 cell and morula), ovaries within each follicular phase were categorized into three groups as described previously (no development, less than average and more than average development). No effect of the stage of embryonic development was detected, that is, the echotexture endpoints of the subordinate follicles from ovaries whose COC produced embryos or did not produce embryos, were not different when examined on specific days of the follicular wave.

4. Discussion

In the present study, computer-assisted quantitative echotexture analysis was used to study the relationship between ultrasound image characteristics, functional status of a follicle, developmental competence of the contained oocyte, and local influence of the CL and

dominant follicle. The results of the spot and line analyses supported the hypothesis that echotexture characteristics of portions of the follicular wall reflect the phase of development of the follicle. We concluded that there was a difference among follicles at different stages of the wave. Changes were evident by Day 7 of the follicular wave when the walls of both dominant and subordinate follicles became hypoechoic.

Changes in the mean grey-scale values of the follicular antrum, studied with the spot analysis technique, were influenced by the day of the follicular wave and follicle type (dominant or subordinate). Both dominant and subordinate follicles collected at Day 7 of the wave had quantitatively darker antra than all the other days examined (Table 1). In an earlier in vivo study (Tom et al., 1998b), no changes were detected in the mean grey-scale values of the antrum in dominant follicles during the follicular wave. However, present findings are supported by another study where both the phase of the follicular wave and the type of follicle (dominant or subordinate) influenced the mean grey-scale values of in vitro acquired high-resolution images of the follicular antrum (Singh et al., 1998). In the latter and present studies, subordinate follicles had brighter antra than dominant ones.

Line analysis of the follicular wall showed lower peak grey-scale values on Days 2 and 7 than on Days 3 and 5 for both subordinate and dominant follicles (Fig. 2). Values of mean grey-scale and the area under the curve decreased significantly between Days 3 and 5 for the subordinate follicles and between Days 5 and 7 for dominant follicles (Figs. 3 and 4). When subordinate follicles were examined histologically (Singh and Adams, 2000), the thickness of the granulosa layer decreased between the late-growing or early-static phase (Day 3) to the regressing phase (Day 6). By design, we utilized the same thickness as the region of analysis for the follicular wall among days. It is reasonable to assume that as the follicular wall became thinner, more of the perifollicular stroma was included in the region of analysis of the follicular wall.

Results of the line analysis of the perifollicular stroma showed that mean grey-scale value, peak grey-scale value and area under the curve decreased with the onset of the static phase for dominant follicles and the regressing phase for subordinate follicles. The echotexture values were lower on Day 7 than on Days 2, 3 or 5 (Figs. 2–4). Conversely, in an earlier study, mean grey-scale values of perifollicular stroma during the late static and regressing phases of both dominant and subordinate follicles were higher than during earlier phases (Singh et al., 1998). Although not significant until Day 7 of the wave, the decreasing trend started at Day 3 for the subordinate follicle stroma, which was delayed until Day 5 for the perifollicular stroma of dominant follicles. A reason for the apparent differences in results around Day 7 of the follicular wave between the two studies (present study and Singh et al., 1998) may be attributed to the difference in the perifollicular vascular flow (in vivo versus in vitro) and/or the quality of images (differences in ultrasound equipment).

Apparent changes in echotexture endpoints of dominant versus subordinate follicles occurred between Days 2 and 3. The peak grey-scale values, area under the curve, and mean grey-scale values were numerically higher for the dominant follicle than subordinates on Day 2; this trend reversed on all subsequent days (Figs. 2–4). These changes, although not statistically significant, occurred at the critical time of selection of the dominant follicle (Adams, 1999). With improvements in ultrasound imaging technology, such changes in echotexture may be used in a diagnostic setting to discriminate between follicles at the time of selection. We speculate that these early echotexture changes occur after (not before) the

detected emergence of the follicular wave because no biochemical or ultrasound differences have been reported among follicles prior to the time of selection (Ko et al., 1991; Adams et al., 1992, 1993; Bergfelt et al., 1994).

In the present study, image data were collected during periods in which dramatic changes in output of luteal and follicular products were expected. Hence, we had the opportunity to investigate the local effect of the CL and the dominant follicle on echotexture characteristics. We concluded that the local presence of the CL and/or the dominant follicle in the ovary did not influence echotexture characteristics of subordinate follicles in the same ovary, and our hypothesis that follicular echotexture characteristics are related to presence of the CL and/or the dominant follicle in the ovary was not supported. This result was supported by other findings obtained in our laboratory (Vassena, 2001) and elsewhere (Ginther et al., 1989c; Sirois and Fortune, 1988) in which ovarian structures did not appear to exert a local effect on the ovary.

Oocyte quality is of paramount importance in assisted reproduction techniques. Finding an echotexture attribute that provides us with the ability to distinguish between competent and incompetent oocytes could provide an important and non-invasive tool for researchers and clinicians. Results of another study in our laboratory showed that oocytes collected from subordinate follicles at Day 5 of the follicular wave were more competent than oocytes collected from Days 2, 3 or 7 of the wave (Vassena, 2001). Interestingly, the differences between the values for the dominant and the subordinate follicles for all the segments of the follicle examined (peripheral antrum, follicle wall and perifollicular stroma) were lowest at Day 5. This observation may be an important link between oocyte competence and echotexture characteristics of the follicle.

Oocytes from subordinate follicles collected at Day 7 displayed lower developmental competence in vitro than those collected on Day 5 (Vassena, 2001). Also, both line and spot analyses revealed lower values for subordinate follicles on Day 7 than on any other day. Thus, it may become possible to use image analysis to monitor the development of follicles during ovarian superstimulation, and to assess the optimal time for oocyte collection for minimizing the effect of a pre- or post-mature follicular environment.

On a subset of the ovaries, we tested the ability of oocytes from subordinate follicles to become fertilized (cleavage stage), undergo the first four mitotic divisions (use the maternal products accumulated in their cytoplasm before major genomic activation), and develop to the morula stage (to test the oocyte ability to switch from maternal to embryonic control of genomic activity and to develop successfully past the 8–16 cell developmental block; Latham, 1999). Echotexture endpoints used in the present study were not associated with the ability to cleave or the proportion of embryos that developed into morulae. However, when we considered development to the 8–16 cell stage, the perifollicular stroma of follicles from ovaries containing developmentally competent oocytes was brighter than the stroma of follicles from ovaries containing incompetent oocytes. This trend was consistent in all echotexture endpoints concerning the stroma. Day 7 subordinate follicles are darker (present study), and less competent (Vassena, 2001) than on earlier days of follicular wave.

In summary, our results from image analysis of perifollicular stroma supported the hypothesis that echotexture is related to the developmental competence of the oocytes contained in the follicle. Subordinate follicles from ovaries that produced 8–16 cell embryos had different echotexture characteristics for the peripheral stroma than follicles from ovaries

that did not produce embryos. The presence of the CL and/or the dominant follicle in the ovary had no effect on echotexture characteristics of the subordinate follicles. We found a close relationship between follicle developmental stage and echotexture analyses of in vivo acquired ultrasound images. Together with the observation that oocyte competence is associated with follicular status (Salamone et al., 1999; Vassena, 2001), results provide important rationale for the use of ultrasound image analysis for identifying follicles which will produce competent oocytes. The sensitivity of this technique is not yet sufficient for use in a diagnostic setting; however, the identification of statistically significant endpoints will form the basis for further improvement of image analysis techniques, with the aim of providing reproductive scientists with a non-invasive, safe and immediate diagnostic tool.

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References

Adams, G.P., 1999. Comparative patterns of follicle development and selection in ruminant. J. Reprod. Fertil. Suppl. 54, 17–32.

Adams, G.P., Matteri, R.L., Kastelic, J.P., Ko, J.C.H., Ginther, O.J., 1992. Association between surges of follicle-stimulating hormone and the emergence of follicular waves in heifers. J. Reprod. Fertil. 94, 177–188.

Adams, G.P., Kot, K., Ginther, O.J., 1993. Effect of the dominant follicle on regression of follicular growth in heifers. Anim. Reprod. Sci. 30, 259–271.

Albertini, D.F., Combelles, C.M., Benecchi, E., Carabatsos, M.J., 2001. Cellular basis for paracrine regulation of ovarian follicle development. Reproduction 121, 647–653.

Bavister, B.D., Yanagimachi, R., 1977. The effects of sperm extracts and energy sources on the motility and acrosome reaction of hamster spermatozoa in vitro. Biol. Reprod. 16, 228–237.

Bavister, B.D., Leibfried, M.L., Lieberman, G., 1983. Development of preimplantation embryos of the golden hamster in a defined culture medium. Biol. Reprod. 28, 235–247.

Bergfelt, D.R., Lightfoot, K.C., Adams, G.P., 1994. Ovarian synchronization following ultrasound-guided transvaginal follicle ablation in heifers. Theriogenology 42, 895–907.

Berisha, B., Pfaffl, M.W., Schams, D., 2002. Expression of estrogen and progesterone receptors in the bovine ovary during estrous cycle and pregnancy. Endocrine 17, 207–214.

Chun, S.Y., Hsueh, A.J., 1998. Paracrine mechanisms of ovarian follicle apoptosis. J. Reprod. Immunol. 39, 63–75.

Farin, P.W., Crosier, A.E., Farin, C.E., 2001. Influence of in vitro systems on embryo survival and fetal development in cattle. Theriogenology 55, 151–170.

Findlay, J.K., Drummond, A.E., Dyson, M., Baillie, A.J., Robertson, D.M., Ethier, J.F., 2001. Production and actions of inhibin and activin during folliculogenesis in the rat. Mol. Cell. Endocrinol. 180, 139–144.

Gandolfi, F., Luciano, A.M., Modina, S., Ponzini, A., Pocar, P., Armstrong, D.T., Lauria, A., 1997. The in vitro developmental competence of bovine oocytes can be related to the morphology of the ovary. Theriogenology 48, 1153–1160.

Ginther, O.J., 1995. Ultrasonographic Imaging and Animal Reproduction: Fundamentals, Book 1. Equiservices Publishing, Cross Plains, WI, USA, pp. 56–57, 69, 165.

Ginther, O.J., Knopf, L., Kastelic, J.P., 1989a. Temporal association among ovarian events in cattle during oestrous cycles with two and three follicular waves. J. Reprod. Fertil. 87, 223–230.

Ginther, O.J., Knopf, L., Kastelic, J.P., 1989b. Ovarian follicular dynamics in heifers during early pregnancy. Theriogenology 41, 247–254.

Ginther, O.J., Kastelic, J.P., Knopf, L., 1989c. Intraovarian relationships among dominant and subordinate follicles and the corpus luteum in heifers. Theriogenology 32, 787–795.

Hazeleger, N.L., Hill, D.J., Stubbings, R.B., Walton, J.S., 1994. Relationship of morphology and follicular fluid environment of bovine oocytes to their developmental potential in vitro. Theriogenology 43, 509–552.

Knopf, L., Kastelic, J.P., Schallenberger, E., Ginther, O.J., 1989. Ovarian follicular dynamics in heifers: test of two-wave hypothesis by ultrasonically monitoring individual follicles. Domest. Anim. Endocrinol. 6, 111–119.

Ko, J.C.H., Kastelic, J.P., Ginther, O.J., 1991. Effect of dominant follicle on ovarian follicular dynamics during the estrous cycle in heifers. J. Reprod. Fertil. 91, 511–519.

Kremkau, F.W., 1998. Diagnostic Ultrasound: Principles and Instruments, 5th ed. Saunders, Philadelphia, PA, USA, pp. 167–168.

Latham, K.E., 1999. Mechanisms and control of embryonic genome activation in mammalian embryos. Int. Rev. Cytol. 193, 71–124.

Lonergan, P., Monaghan, P., Rizos, D., Boland, M.P., Gordon, I., 1994. Effect of follicle size on bovine oocyte quality and developmental competence following maturation, fertilization and culture in vitro. Mol. Reprod. Dev. 37, 48–53.

Madison, V., Avery, B., Greve, T., 1992. Selection of immature bovine oocytes for developmental potential in vitro. Anim. Reprod. Sci. 27, 1–11.

Pierson, R.A., Adams, G.P., 1995. Computer-assisted image analysis, diagnostic ultrasonography and ovulation induction: strange bedfellows. Theriogenology 43, 105–112.

Pierson, R.A., Ginther, O.J., 1987. Follicular populations during the estrous cycle in heifers. II. Influence of right and left sides and intraovarian effect of the corpus luteum. Anim. Reprod. Sci. 14, 177–186.

Pierson, R.A., Ginther, O.J., 1988. Follicular populations during the estrous cycle in heifers. III. Time of selection of ovulatory follicle. Anim. Reprod. Sci. 16, 81–95.

Rosenkrantz Jr., C.F., Zeng, G.Z., Schoff, P.K., First, N.L., 1990. A simple medium for in vitro development of bovine embryos. J. Anim. Sci. 69 (Suppl. 1), 430.

Salamone, D.F., Adams, G.P., Mapletoft, R.J., 1999. Changes in the cumulus-oocyte complex of subordinate follicles relative to follicular wave status in cattle. Theriogenology 52, 549–561.

Savio, J.D., Keenan, L., Boland, M.P., Roche, J.F., 1988. Pattern of growth of dominant follicles during the estrous cycle of heifers. J. Reprod. Fertil. 83, 663–671.

Singh, J., Adams, G.P., 2000. Histomorphometry of dominant and subordinate bovine ovarian follicles. Anat. Rec. 258, 58–70.

Singh, J., Pierson, R.A., Adams, G.P., 1997. Ultrasound image attributes of the bovine corpus luteum: structural and functional correlates. J. Reprod. Fertil. 109, 35–44.

Singh, J., Pierson, R.A., Adams, G.P., 1998. Ultrasound image attributes of bovine ovarian follicles and endocrine and functional correlates. J. Reprod. Fertil. 112, 12–29.

Sirois, J., Fortune, J.E., 1988. Ovarian follicular dynamics during the estrous cycle and early pregnancy in ewes. Biol. Reprod. 39, 308–317.

Tom, J.W., Pierson, R.A., Adams, G.P., 1998a. Quantitative echotexture analysis of bovine corpora lutea. Theriogenology 49, 1345–1352.

Tom, J.W., Pierson, R.A., Adams, G.P., 1998b. Quantitative echotexture analysis of bovine ovarian follicles. Theriogenology 50, 339–346.

Vassena R., 2001. Bovine oocyte competence. M.Sc. Thesis. University of Saskatchewan, Saskatoon, Sask., Canada.